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Evaluation of in vitro activity of five antimicrobial agents on *Acanthamoeba* isolates and their toxicity on human corneal epithelium

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BACKGROUND: *Acanthamoeba* keratitis (AK) is an important cause of ocular morbidity in both contact lens wearers and non wearers. Medical management comprises prolonged empiric treatment with multiple drugs, leading to adverse effects and suboptimal cure. The present study evaluated the efficiency and safety of common antimicrobial agents used in treatment of AK.

METHODS: Six *Acanthamoeba* isolates (four AK, two water samples) were axenized and subjected to in vitro susceptibility testing against chlorhexidine, pentamidine isethionate, polymyxin B, miltefosine, and fluconazole to check for trophocidal and cysticidal activity. The safety profile was analysed by observing the cytotoxicity of the highest cidal concentration toward human corneal epithelial cell (HCEC) line.

RESULTS: Chlorhexidine had the lowest cidal concentration against both cysts and trophozoites (range 4.16–25 µg/ml) followed by pentamidine isethionate (range 25–166.7 µg/ml). Both agents were nontoxic to HCEC. Polymyxin B (range 25–200 µg/ml) and fluconazole (range 64–512 µg/ml) had relatively higher minimum inhibitory concentrations (MIC); fluconazole was nontoxic even at 1024 µg/ml, but cytotoxicity was observed at 400 µg/ml with polymyxin B. Miltefosine was not effective against cysts at tested concentrations. *A. castellanii* were more susceptible to all agents (except pentamidine isethionate) than *A. lenticulata*. Clinical isolates were less susceptible to polymyxin B and fluconazole than environmental isolates, reverse was true for miltefosine.

CONCLUSION: Chlorhexidine and pentamidine isethionate were the most effective and safe agents against both trophozoites and cysts forms of our *Acanthamoeba* isolates. Fluconazole had higher MIC but was nontoxic. Polymyxin B was effective at high MIC but therapeutic dose was found toxic. Miltefosine, at tested concentrations, could not inhibit cysts of *Acanthamoeba*. Clinical isolates had higher MICs for polymyxin B and fluconazole.

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INTRODUCTION

Acanthamoeba keratitis (AK), the corneal infection caused by pathogenic free-living amoeba belonging to genus *Acanthamoeba*, is a severe and potentially sight-threatening condition [1]. Though AK was first reported in early 1970s, a steep increase in incidence of AK was observed in 1980s parallel to the usage of soft contact lenses (CL) [2] so much so that Centers for Disease Control and Prevention (CDC) issued an advisory in 1985 to alert the ophthalmologists regarding the association of AK with use of CL [3]. The factors contributing to this association include a combination of corneal microtrauma, suboptimal CL hygiene, exposing CL to contaminated water environments and improper storage [2]. Another more traditional route of infection, the predominating risk factor for AK in Asian countries [4] and rural settings [5], is the exposure of an injured corneal epithelium to water or soil contaminated with *Acanthamoeba*. Thus, environmental contamination, especially that via water containing *Acanthamoeba* [6], is an important risk factor for AK for both CL- and non-CL wearers. AK has also been reported following invasive

corneal surgery [7] and there is one reported case where no risk factor could be identified [8].

The global incidence of AK is difficult to ascertain as not only the incidence varies with geographical area and risk factor involved, but the clinical presentation of AK also mimics those of other infectious keratitis, especially herpetic and fungal [9]. Though a tentative diagnosis of AK can be made by visualizing the typical parasite using either in vivo confocal microscopy [10] or examining the corneal scraping under microscope, culture remains the gold standard for microbiological diagnosis [11]. Culture being cumbersome and time-consuming, PCR-based identification is being widely used as a rapid and sensitive approach for AK diagnosis [12].

The management of AK encompasses timely diagnosis and achieving medical cure with restoration of corneal transparency. Penetrating keratoplasty is done in elective conditions for optical indications [2, 9]. To achieve a medical cure, a variety of drugs are usually started empirically and given for long durations of time extending for months together [13]. However, since

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Acanthamoeba can biologically shift between trophozoite and cyst forms, not all drugs can effectively clear the infection as either the cyst form is resilient to the usual dosage of drug or the dosage required for clearance is too toxic for corneal epithelium [14]. Thus, in vitro susceptibility testing (IVST) of potential therapeutic agents against both morphological forms of *Acanthamoeba* may contribute to customized therapy for the patient of AK, thereby leading to favorable clinical outcomes. Though several studies have evaluated the in vitro susceptibility of varied drugs on AK isolates [15–18], these studies are geographically limited and have employed varied parameters for end-result evaluation. To the best of our knowledge, other than a study by Narsimhan et al. [19] that evaluated minimum cysticidal concentration (MCC) for two antiparasitic agents in year 2002, there is no Indian study evaluating IVST for AK isolates. The present study was therefore designed to systematically carry out IVST of *Acanthamoeba* isolates from AK and environmental sources to determine the therapeutic options for Indian isolates. Miltefosine, the repurposed drug being increasingly used for AK, granulomatous amoebic encephalitis and fungal keratitis, was also evaluated along with other conventional drugs. Further, host cytotoxicity assays were conducted to evaluate safety of use for the resultant effective drug concentrations. To the best of our knowledge, such cytotoxicity assays on human corneal epithelial cell (HCEC) lines have not been performed for anti-acanthamoebic drugs.

MATERIALS AND METHODS

Acanthamoeba strains

Out of 300 clinically-suspected cases of AK presenting to our tertiary care center over a period of 4 years (2014–2018), 11 cases were confirmed as AK by 18S rDNA sequencing, as described previously for granulomatous amoebic encephalitis isolates [20]. Further, from 100 water samples (tap water or swabs from inner side of tap mouth) collected from various intensive care units of our institute in the year 2014, four were molecularly confirmed to be containing *Acanthamoeba* spp [21]. Although nine out of 11 AK isolates and all four water isolates could be cultured on NNA medium, only four AK and two water isolates could be axenized and maintained for carrying out all IVST experiments. Among the four AK isolates, two were *A. castellanii* (T4), 1 *A. lenticulata* (T5), and 1 *A. hatchetti* (T3). Between the two water isolates, one was *A. castellanii* and other was *A. lenticulata*. The study was approved by the Institute Ethics Committee (INT/IEC/2017/674).

Maintenance of *Acanthamoeba* isolates

All the six *Acanthamoeba* isolates were slowly adapted to axenic culture without bacteria in improvised PYG media supplemented with RNAase and NCTC109 vitamin mixture till *Acanthamoeba* trophozoite monolayer was obtained [22]. For encystment, *Acanthamoeba* trophozoites in axenic culture were inoculated onto non-nutrient agar plates without *Escherichia coli*. The NNA plates were incubated at 30 °C for 7 days. After encystment the actively multiplying trophozoites were converted to cysts and were then collected in 1 ml of PBS and stored at 4 °C for drug susceptibility testing.

In vitro drug susceptibility testing

Procedure. The *Acanthamoeba* isolates were tested for their susceptibility to the following drugs: chlorhexidine, pentamidine isethionate, polymyxin B sulfate, miltefosine, and fluconazole (all purchased from Sigma Aldrich, India). The drug susceptibility testing were designed to determine minimal motility inhibition concentration (MMIC), minimal inhibitory concentration (MIC), and MCC of the drugs. Briefly, the stock solutions of all the therapeutic drugs were prepared in peptone yeast dextrose medium and subsequently twofold serial dilutions were made. The numbers of cysts or trophozoites were counted in a hemocytometer and suspension was adjusted to a final concentration of 10⁴ cysts or trophozoites/mL. Four hundred microliter of each drug concentration was placed in 24 well tissue culture plate (Greiner Bio-one, Germany). One hundred microliter suspensions containing 10⁴ trophozoites/ml were added to each well and were incubated at 30 °C for 6, 24, and 48 h, as described previously [23]. The positive control was trophozoite/cysts mixed with PBS and

negative control contains only drug without the addition of the trophozoite/cysts. The plates were incubated for a total of 48 h and read at 6 h for MMIC determination, and at 24 h and 48 h for MIC50 and MIC90 determination. The in vitro drug susceptibility testing was carried out in triplicate in three different sets of experiments and subjective assessment was performed by a trained microscopist. Means were calculated for the 6 h—MMIC, 24 h and 48 h MIC50 and MIC90, and MCC against each strain for every drug.

Interpretation

MMIC-6 h: The MMICs at 6 h were visually observed by microscopic examination and compared with controls. The MMIC was defined as the minimum concentration of drug that inhibits pseudopodial activity as compared to controls and is characterized by rounding up, diminution in size, and increase in granulation [23].

MIC50-24, MIC50-48, MIC90-24, and MIC90-48 h: The MIC50 and MIC90 was defined as the minimum concentration of drug at which growth was <50% and 90%, respectively, as compared to control, as observed by microscopic examination [23].

MCC: MCC was defined as the minimum concentration of drug needed to prevent trophozoite formation after 14 days of incubation. The twofold drug dilutions were made for each drug, 100 µl of cysts at a concentration of 10⁴ cysts/ml was added and the tubes were incubated at 30 °C for 48 h. After 48 h of incubation, the tubes were centrifuged at 5000 × g for 5 min. The supernatant of drug solution were removed and deposited cysts were washed twice with PBS to remove the drug. The NNA plates with *E.coli* were labeled and final washed cysts were inoculated on respective plate including controls and incubated at 30 °C. Plates were first examined after 2 days and then daily till 14 days for the presences of trophozoites. Results were compared to positive control without drug which show presence of trophozoites after 48 h of incubation [19].

Host cytotoxicity assay

Cell culture. The HCEC were grown in 25 cm² culture flasks (Greiner Bio-one, Frickenhausen, Germany). The cells were cultured in Dulbecco's Modified Eagle's Medium DMEM/F-12 1:1 (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B (sigma) in humidified environment at 37 °C with 5% CO₂. The cell lines were allowed to grow for 48–72 h. The growth medium was replaced with fresh growth medium every second day. After formation of confluent monolayers (examined under inverted phase contrast microscope), the cells were sub cultured by splitting with trypsin EDTA.

Cytotoxicity assay. The MTT assay was performed to determine the cell cytotoxicity of all the therapeutic drugs for Human corneal cell line (HCEC). The evaluation is based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells, to give a blue formazan product that is measured spectrophotometrically. The MTT was dissolved in the growth medium as 5 mg/ml stock solution.

The HCEC cells (1 × 10⁵/ml) were seeded in 96 well microplates and incubated at 37 °C overnight with 5% CO₂ for attachment. After 24 h, when 90% confluence was achieved, freshly prepared dilutions of all the therapeutic drugs in growth medium were added to the each well and the cultures were further incubated. After 6, 12, 24, and 48 h, the cells were washed with 1 × sterile PBS and 20 µl freshly prepared working solution of MTT was added for a further incubation period of 3 h. After 3 h, the MTT solution was removed and 100 µl of DMSO was added in both treated and non-treated wells to dissolve the formazan and incubated for 15 min at 37 °C with shaking. Optical density was measured spectrophotometrically at 596 nm with 620 nm as reference wavelength. Cell line without drug was taken as positive control and cells without MTT as negative control. Percentage of cell cytotoxicity was calculated as:

$$\% \text{ cytotoxicity concentration} = \frac{\text{OD of control} - \text{OD of treated wells}}{\text{OD of Control}} \times 100$$

The acceptable limit of cytotoxicity was taken as the viability cutoff of 60%, as described previously [24], thus a cytotoxicity of >40% was considered unacceptable.

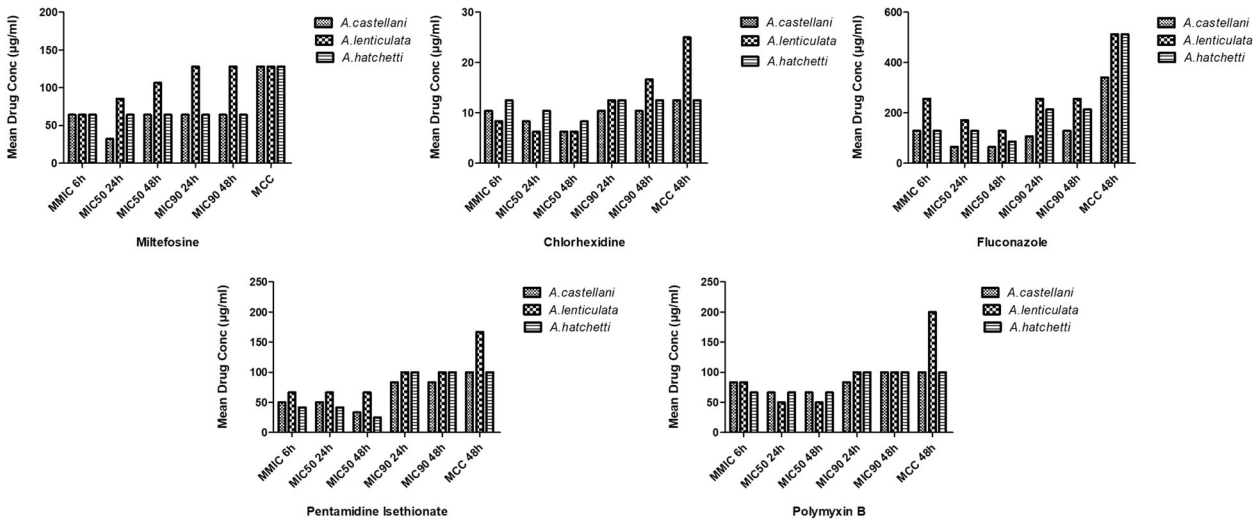


Fig. 1 Graphical representation comparing the in vitro susceptibility of four clinical isolates of *Acanthamoeba* against five different drugs. The isolates consisted of *A. castellanii* ($n = 2$), *A. lenticulata* ($n = 1$) and *A. hatchetti* ($n = 1$). The susceptibility was evaluated against miltefosine, chlorhexidine, fluconazole, pentamidine isethionate and polymyxin B. The x-axis represents the six parameters used to test susceptibility and the y-axis represents the mean drug concentration.

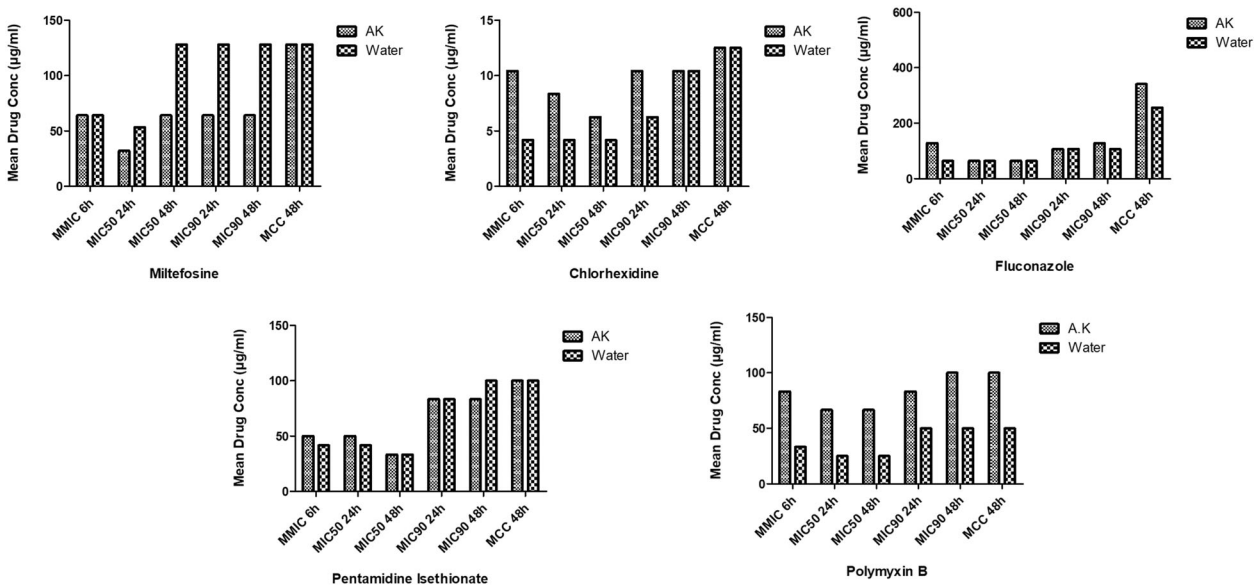


Fig. 2 Graphical representation comparing the in vitro susceptibility of *Acanthamoeba castellanii* isolated from *Acanthamoeba keratitis* patients ($n = 2$) with environmental isolate ($n = 1$). The x-axis represents the six parameters used to test susceptibility and the y-axis represents the mean drug concentration.

RESULTS

In vitro activity of anti-Acanthamoebic drugs (Figs. 1, 2, 3)

Chlorhexidine. Among all the tested drugs, chlorhexidine was effective at the lowest concentrations for all parameters. It showed both trophocidal and cysticidal activity. The environmental *A. castellanii* (4.16 µg/ml; $N = 1$) had lower MMIC-6 h than clinical *A. castellanii* (10.42 µg/ml; $N = 2$). The MIC90 was lower for *A. castellanii* as compared to *A. lenticulata* for the environmental isolates [(MIC90-24 h) 6.25 µg/ml vs. 12.5 µg/ml, $N = 1$ for each] and clinical isolates [(MIC90-48 h) mean 10.42 µg/ml, $N = 2$; vs. 16.67 µg/ml; $N = 1$] strains. The MCC among AK isolates was lower for *A. castellanii* (mean 12.5 µg/ml; $N = 2$) and *A. hatchetti* (12.5 µg/ml; $N = 1$) as compared to *A. lenticulata* (25 µg/ml; $N = 1$), while no such difference was observed between water samples.

Pentamidine isethionate. The trophocidal activity of pentamidine isethionate was nearly uniform for all isolates, irrespective of their origin (clinical or environmental) and genotype. With regard to cysticidal activity, the MCC of pentamidine isethionate was higher for clinical strain of *A. lenticulata* (166.7 µg/ml; $N = 1$) as compared to both *A. castellanii* (mean 100 µg/ml; $N = 2$) and *A. hatchetti* (100 µg/ml; $N = 1$). No such difference was observed between environmental isolates.

Polymyxin B sulfate. With regard to trophocidal activity, similar response was observed among clinical isolates, irrespective of genotype. The trophocidal activity among environmental isolates varied with the genotype; *A. castellanii* was more susceptible than *A. lenticulata* for all parameters tested except MMIC-6 h. Among *A.*

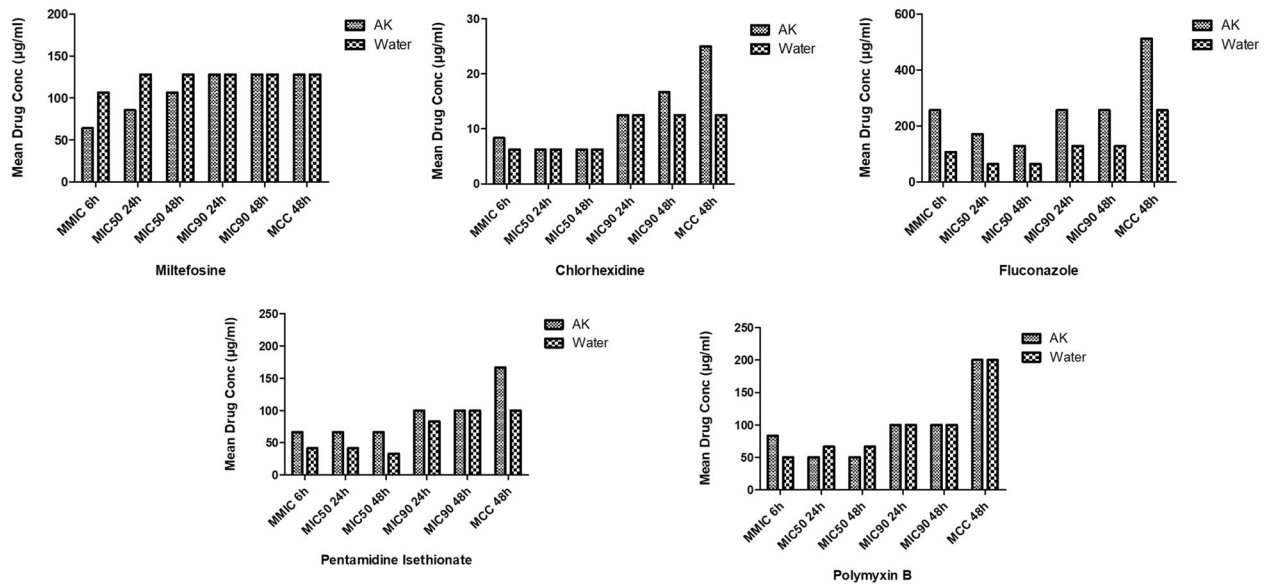


Fig. 3 Graphical representation comparing the in vitro susceptibility of *Acanthamoeba lenticulata* isolated from *Acanthamoeba keratitis* patient ($n = 1$) with environmental isolate ($n = 1$). The x-axis represents the six parameters used to test susceptibility and the y-axis represents the mean drug concentration.

castellanii isolates, clinical strains were less susceptible than environmental strains for all the parameters tested except MIC90-24 h. With regard to cysticidal activity, *A. lenticulata* (200 µg/ml; $N = 1$) was least susceptible to polymyxin B, irrespective of source.

Miltefosine. With regard to trophocidal activity, miltefosine showed better activity against clinical isolates for both the genotypes than their corresponding environmental isolates; i.e., clinical *A. castellanii* was more susceptible than environmental *A. castellanii*, and clinical *A. lenticulata* was more susceptible than environmental *A. lenticulata* against the parameters MMIC-6 h and MIC50. For MIC90, this difference was observed only for *A. castellanii*. Even among the clinical strains, the MIC at 24 h (both MIC50-24 h and MIC90-24 h) was lower for *A. castellanii* than other two genotypes. The cysticidal activity of miltefosine was poor with all strains having MCC of 256 µg/ml, irrespective of source and genotype.

Fluconazole. The trophocidal activity of fluconazole varied with the source as well as genotype. While clinical isolates of *A. castellanii* were more susceptible to fluconazole than *A. lenticulata* or *A. hatchetti* for all parameters tested, no such difference was observed among environmental strains for any of the parameters. Further, with respect to genotype *A. lenticulata* per se, clinical isolates were less susceptible than environmental isolates for all parameters. The cysticidal activity for fluconazole was observed at relatively higher concentrations than all other drugs tested. Among clinical isolates, MCC was lower for *A. castellanii* (mean 341.3 µg/ml; $N = 2$) as compared to other two genotypes (512 µg/ml; $N = 1$ for each); no difference among environmental isolates was observed. With regard to genotype, cysts of *A. lenticulata* from clinical samples (MCC 512 µg/ml; $N = 1$) were more resilient to fluconazole than those recovered from water samples (MCC 256 µg/ml; $N = 1$).

The MIC of each isolate against each parameter and drug, when done in triplicate, was within ± 1 twofold dilution, thus vouching for repeatability of assay.

Host cytotoxicity analysis

The cytotoxic effect of different drugs, as tested on HCEC using MTT assay, is depicted in Table 1. At observed effective drug

concentrations, chlorhexidine and polymyxin B were least toxic to HCEC cells with 24.67% and 24.89% cytotoxicity, respectively, at 24 h and 25.8% and 27% cytotoxicity, respectively, at 48 h. The cytotoxicity of pentamidine isethionate at observed cidal concentrations was 26.78% at 24 h and 28.9% at 48 h. Fluconazole exhibited toxicity of 31.61% for observed cidal concentration at 24 h and 29.18% at 48 h. For miltefosine, the cytotoxicity could not be evaluated as the observed cidal concentration was 256 µg/ml and the range of drug concentration tested was 2–128 µg/ml.

On evaluating the cytotoxicity of these drugs at the highest concentration tested, chlorhexidine had the least toxicity of 29.45% at 24 h and 32.34% at 48 h. Pentamidine isethionate, at highest concentration of 400 µg/ml, had acceptable toxicity of 39.29% at 24 h which increased to unacceptable toxicity of 44.67% at 48 h. The toxicity of polymyxin B, at highest tested concentration of 400 µg/ml, was >40% at both times with highest toxicity of 46.67% at 48 h. The cytotoxicity of fluconazole at highest tested concentration of 1024 µg/ml was marginally above 40% at both the times and that for miltefosine at highest tested concentration of 128 µg/ml was ~37% at both the times.

DISCUSSION

AK is a therapeutic challenge with potential for treatment failure. With no specific drug being approved for AK by Food Drug and Administration [9], various antimicrobials are given in different combinations for different durations to increase the likelihood of successful treatment. The treatment is to be continued for prolonged periods of time even after clinical resolution of active infection as the drugs are less active against the cystic forms and untimely stoppage of medication can result in relapse [9]. Further, since *Acanthamoeba* are eukaryotes and share several functional homologies with mammalian cells, many of the drugs cannot be prescribed at their effective concentrations due to untoward side effects. Therefore, as rightly suggested by Siddiqui et al. [25] in their elaborate review, a “targeted therapeutic approach” should be developed based on the mechanism of action of the drugs so as to harness their maximum antiparasitic effect without concomitant host damage. Broadly, anti-AK drugs can be grouped as membrane-acting agents [like biguanides (chlorhexidine), antibacterial agents (polymyxin B), and antifungal agents

Table 1. Cytotoxicity of the tested drugs as evaluated by exposing HCEC cell lines to observed cidal concentration and highest tested concentration for 24- and 48 h.

| Drug | Range tested $\mu\text{g/ml}$ | Highest observed cidal concentration $\mu\text{g/ml}$ | | Cytotoxicity % after 24 h exposure | | Cytotoxicity % after 48 h exposure | |
|------------------|-------------------------------|-------------------------------------------------------|------------|------------------------------------|-------------------------|------------------------------------|-------------------------|
| | | Trophocidal | Cysticidal | Cidal Conc. | At highest tested conc. | At observed cidal conc. | At highest tested conc. |
| Chlorhexidine | 0.78–50 | 16.67 | 25 | 24.7 | 29.45 | 25.8 | 32.34 |
| Pent Isethionate | 6.25–400 | 100 | 166.7 | 26.78 | 39.29 | 28.9 | 44.67 |
| Polymyxin B | 6.25–400 | 100 | 200 | 24.89 | 43.98 | 27 | 46.67 |
| Miltefosine | 2–128 | >128 | >128 | ND | 36.23 | ND | 38.23 |
| Fluconazole | 16–1024 | 256 | 512 | 31.61 | 40.74 | 29.18 | 42.76 |

Conc. Concentration, ND not determined.

(fluconazole)], nucleic acid acting agents (pentamidine isethionate), and intracellular targeting agents (miltefosine) [25]. For the present study on in vitro evaluation of susceptibility of *Acanthamoeba*, we intentionally selected agents representing each of these mechanisms of action. The commonly used drug polyhexamide (PHMB) was intentionally not evaluated as the phase III ODAK (Orphan drugs for AK) clinical trial (European Clinical Trial 2016-001823-30) addressing its efficacy and safety profile is underway since 2017.

In the current study, the drug with highest anti-acanthamoebic activity was chlorhexidine. With an MIC ranging from 4.16 to 16.67 $\mu\text{g/ml}$ and MCC ranging from 12.5 to 25 $\mu\text{g/ml}$, it showed potent trophocidal and cysticidal activity. *A. castellanii* strains were more susceptible than *A. lenticulata*, and environmental strains were more susceptible than AK strains. Single study by Padzik et al. [26] reported environmental *A. castellanii* Neff strain to be more resilient to chlorhexidine than clinical *A. polyphaga* from AK patient. Since no other study on anti-acanthamoebic drugs has compared in vitro activity of AK strains with environmental strains or inter-genotypic evaluation, a direct comparison with available literature is not possible. With regard to trophocidal activity of chlorhexidine, the MIC of our isolates lie well within the range reported in previous studies. Elder et al. [16] in their pioneering work on 23 AK isolates from London in 1994, reported mean MIC of 0.7 $\mu\text{g/ml}$ for chlorhexidine and Lim et al. [15] reported mean MIC of 32 $\mu\text{g/ml}$ (ranging from 16 to 64 $\mu\text{g/ml}$) for their 19 Australian AK isolates. However, while Elder et al. [16] have not reported genotypic characterization of isolates, Lim et al. [15] included one *A. castellanii* and two *A. hatchetti* strains but have not reported any inter-genotypic comparison. Ortega et al. [17] reported MIC of 4.68 $\mu\text{g/ml}$ for chlorhexidine against one clinical *A. castellanii* strain from AK patient. With regard to cysticidal activity of chlorhexidine, our isolates ($n = 6$) had MCC similar to that reported by Llyod (27 $\mu\text{g/ml}$) [27] and lower than 40 $\mu\text{g/ml}$ reported from Australia in the year 2000 ($n = 19$) [15] and 32.81 $\mu\text{g/ml}$ reported from South India in 2001 ($n = 19$) [19]. This difference could arise due to geographical and genotypic variations among the isolates, and differences in in vitro assays. Interestingly, the mean MCC for chlorhexidine reported by Elder et al. [16] from London in 1994 was 2.77 $\mu\text{g/ml}$ and that by Perez et al. [28] from the same hospital in London, in 2003 was 10.8 $\mu\text{g/ml}$. This suggests that, may be slowly, but MCC to even the most active agent has increased over a decade and hence it becomes important that local susceptibility data is generated as a continuous process.

Chlorhexidine, in the present study, was found to be safe for use at observed cidal concentration (25 $\mu\text{g/ml}$) and highest tested concentration (50 $\mu\text{g/ml}$) with cytotoxicity ranging from 24 to 32%. For the treatment of AK, chlorhexidine is used as a 0.02% topical agent, i.e., concentration of 200 $\mu\text{g/ml}$. Although the available world literature has reported highest MIC or MCC of

64 $\mu\text{g/ml}$ for chlorhexidine among AK isolates, the recommended dosage contains a 2–3 higher fold concentration as firstly, it would compensate for the dilution of drug happening by continuous lacrimal fluid flow, and secondly, even at higher concentration than 200 $\mu\text{g/ml}$, chlorhexidine has been found safe [29]. However, the concentration of chlorhexidine (0.003%; 30 $\mu\text{g/ml}$) present in multiple purpose contact lens solution may not be able to kill all *Acanthamoeba* species which may be one of the reasons for predominance of AK in contact lens wearers.

The anti-acanthamoebic activity of pentamidine isethionate, in the current study, varied from MIC of 25 to 100 $\mu\text{g/ml}$ and MCC of 100 to 166.7 $\mu\text{g/ml}$. The MICs of our isolates were higher than those reported earlier, ranging from 0.56 $\mu\text{g/ml}$ in London [16] to 60 $\mu\text{g/ml}$ in Australia [15]. The cysticidal activity of pentamidine isethionate, however, was in concordance with prior studies reporting MCC ranging from 64 to >128 $\mu\text{g/ml}$ [15, 16, 18]. Pentamidine isethionate is clinically used at a cidal concentration of 0.01–0.02% [100–200 $\mu\text{g/ml}$] [25]. On toxicity assay analysis of our study, the highest cidal concentration of 166.7 $\mu\text{g/ml}$ was found to be safe for use, however, the highest concentration tested (400 $\mu\text{g/ml}$) was safe at 24 h but produced >40% toxicity on prolonged exposure of 48 hr. This is in accordance with a study by Alizadeh et al. [30] wherein short term contact of pentamidine isethionate with cell cultures was found safe for use while prolonged exposure caused some damage to superficial epithelium.

Polymyxin B was observed to have MIC ranging from 25 to 100 $\mu\text{g/ml}$ and MCC from 50 to 200 $\mu\text{g/ml}$. A single study [16] evaluating the in vitro susceptibility of polymyxin B as monotherapy, reported a trophozoite minimum amoebicidal concentration of 19.2 $\mu\text{g/ml}$ and a much higher MCC of >500 $\mu\text{g/ml}$. These vast differences could possibly arise from differences in genotypes and the methodology employed for MCC. On cytotoxicity evaluation, while 200 $\mu\text{g/ml}$ was found safe for HCEC cells, the highest tested concentration of 400 $\mu\text{g/ml}$ produced unacceptable toxicity even at 24 h which increased by 48 h. The therapeutic dose for AK is 10,000–25,000 U/ml which corresponds to 1–2.5 mg/ml, and hence 1000–2500 $\mu\text{g/ml}$ [31]. With no studies evaluating its corneal toxicity, extrapolating our results of polymyxin B toxicity make this therapeutic dose appear too toxic for use. Further studies are suggested to define the limits of toxicity for this antibacterial agent.

The MIC for miltefosine ranged from 64 to 256 $\mu\text{g/ml}$ and MCC was 256 $\mu\text{g/ml}$ for all in the present study. This is similar to the observations of Garajova et al. [32] who in their evaluation of four alkylphosphocholines (miltefosine and its analogs) on two AK (*A. castellanii*) isolates observed the MIC to range from 26 to 256 $\mu\text{g/ml}$ and MCC was >256 $\mu\text{g/ml}$. Similarly, Chao et al. [33] in their evaluation of in vitro cysticidal activity of miltefosine against three environmental *Acanthamoeba* isolates, documented MCC of 4.84 mM at day 7 of incubation [corresponding to 2 g/ml]. The ocular

drops of miltefosine contain 65.12 µg/ml. While this concentration was found nontoxic [34] and our experiments have shown that a concentration of 128 µg/ml is also safe, the concentration of miltefosine in therapeutic solution may not kill all trophozoites of *Acanthamoeba* and any cyst at all. Mrva et al. [35] have also cautioned against the clinical efficacy of miltefosine owing to its insusceptibility against cyst forms. It is possible that the greater content of cellulose present in the cyst wall hampers activity of miltefosine [27].

Fluconazole, the last drug included in the present analysis, had MIC range from 64 to 256 µg/ml and MCC range from 256 to 512 µg/ml. This was in accordance with the results of prior two studies wherein MIC of fluconazole was reported to be >320 µg/ml [16] and >1024 µg/ml [36], while MCC was reported as >500 µg/ml [16] and >128 µg/ml [18]. While Hernández-Martínez et al. [37] reported higher in vitro susceptibility to voriconazole in their two *A. castellanii* isolates from Mexican patients, Talbot et al. [38] did not find it cysticidal in any of their ten AK isolates (mean MCC > 10,000 µg/ml). Thus, therapeutic success of AK with fluconazole is questionable. All azoles, other than fluconazole, are lipophilic and hence are poorly absorbed in aqueous medium [39]. Azoles are used as 1% topical agent for fungal ocular infections and the same dosage is extended as anti-acanthamoebic treatment. This concentration is used to cover even those fungal agents that have high MIC for fluconazole. Previous studies, as reviewed by Lakhani et al. [39] have shown fluconazole to be minimally toxic to corneal epithelium even at high concentrations and fluconazole-loaded implants are also used for sustained and prolonged release of the drug. In our study also, minimal cytotoxicity was observed at the highest tested concentration of fluconazole.

Another observation made in the current study was that clinical strains had a general trend of higher MICs to all drugs except miltefosine than their environmental counterparts; especially for polymyxin B and fluconazole (even for MCC). It could be possible that the rampant use of these two drugs in clinical practice for their basic antibacterial and antifungal role, respectively made the clinical isolates less susceptible to them. Interestingly, in miltefosine MIC, reverse phenomenon was noticed i.e., environmental isolates had higher MICs for all the parameters tested than the clinical isolates. This could possible arise due to the conferring of cross-resistance by various phosphocholines and their ammonium derivatives that are used as cleaning agents and disinfectants, thus making the environmental isolates more resilient. The observation that *A. lenticulata* stains had higher MICs than *A. castellanii* irrespective of the source, can be explained by the fact that *A. lenticulata* belong to group III morphotype which have thicker wall [40] conferring protection against physical and chemical treatment.

The study has following limitations: only six isolates of *Acanthamoeba* could be evaluated for in vitro susceptibility. Though nine AK and four water isolates could be successfully cultured, only six out of these 13 could be properly axenized and revived for each experiment. Though our results can help ophthalmologists choose a suitable drug with least toxicity for AK treatment, such small sample size precludes any generalizations and warrants further evaluation in larger prospective studies. While studies on *Acanthamoeba* susceptibility have documented evaluation of a single drug [17] to as many as 31 drugs [18], we used only five that were more clinically relevant and represented all mechanisms of action. Lastly, longer incubation could have been carried out beyond 48 h to evaluate *Acanthamoeba* activity after the drug treatment.

To summarize, chlorhexidine was the most effective agent against our isolates having potent trophocidal and cysticidal activity with no toxic effects. The second best anti-Acanthamoebic agent was pentamidine isethionate with good activity and safety profile. To ensure optimal medical cure, a combination of the two could be used. Polymyxin B and fluconazole could be used a

alternatives especially if concomitant coinfection with bacterial and fungal agents is suspected. However, dosage of polymyxin B appeared too toxic and may be replaced with some other antibacterial agent while fluconazole was well tolerated and could be used as sustained-release depots. Miltefosine had relatively higher MIC and MCC and the therapeutic dose was found insufficient to clear all the trophozoites and any of the cysts. Clinical isolates, in comparison to environmental isolates, had higher MICs for polymyxin B and fluconazole, and lower MICs for miltefosine. Among the clinical isolates, *A. lenticulata* had the highest MICs.

Summary

What was known before

- *Acanthamoeba* keratitis, the corneal infection caused by *Acanthamoeba*, is a severe and potentially sight-threatening condition.
- The treatment is largely empirical and there is an unmet need for evaluating drug susceptibility testing for local strains.
- In vitro susceptibility testing of potential therapeutic agents against both trophozoite and cysts contribute to customized therapy for the patient of AK, thereby leading to favorable clinical outcomes.

What this study adds

- Clinical as well as environmental isolates representing different genotypes of *Acanthamoeba* were evaluated for in vitro drug susceptibility testing using six different parameters (6 hr – MMIC, 24-hr & 48-hr MIC₅₀ and MIC₉₀, and MCC) against five drugs along with cytotoxicity assay on human corneal epithelial cells. Chlorhexidine and pentamidine isethionate were the most effective and safe agents against both trophozoites and cysts forms of our *Acanthamoeba* isolates.
- Fluconazole had higher MIC but was non-toxic.
- Polymyxin B was effective at high MIC but therapeutic dose was found toxic.
- Miltefosine, at tested concentrations, could not inhibit cysts of *Acanthamoeba*.

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Conceptualization: SK, AG, data curation: KM, AG, and CS, experiments: KM, CS, analysis: MS, KM, and SK, draft preparation: MS, KM, revision and editing: KM, MS, and SK, and study supervision: SK, RS.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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