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Antiamoebic susceptibility in *Acanthamoeba* keratitis: comparison of isolates from South India and Northern California

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Abstract

Purpose: Outcomes of *Acanthamoeba* keratitis are often worse in India than in the United States. The goal of the present study was to determine whether antiamoebic susceptibility patterns were different when comparing *Acanthamoeba* isolates from India to those of the US.

Methods: *Acanthamoeba* isolates were obtained from corneal scrapings of 43 patients with infectious keratitis seen at the Francis I. Proctor Foundation (N=23) and Aravind Eye Hospital (N=20) from 2008 through 2012 and plated on growth media. A previously described minimum cysticidal concentration (MCC) assay was performed by a single laboratory technician to assess susceptibility to five anti-amoebic agents for all isolates. Testing was done in triplicate, with the median MCC chosen for analyses.

Results: The MCC (μg/mL) of polyhexamethylene biguanide (PHMB) was 6.25 [IQR 5.47–12.5] for Aravind isolates and 6.25 [IQR 6.25–9.375] for Proctor isolates (p=0.75); corresponding values were 6.25 [IQR 3.125–6.25] and 3.125 [IQR 3.125–9.375] for chlorhexidine (p=0.81); 2500 [IQR 2500–5000] and 5000 [IQR 1250–20000] for voriconazole (p=0.25); 15.6 [IQR 15.6–39.0625] and 15.6 [IQR 15.6–31.25] for hexamidine (p=0.92); and 15.6 [IQR 7.81–15.6] and 15.6 [IQR 7.81–31.25] for propamidine (p=0.42).

Conclusions: This study found no statistically significant differences in antiamoebic susceptibility of Indian vs. US samples from *Acanthamoeba* keratitis clinical isolates. These findings suggest that differences in antiamoebic susceptibility are likely not responsible for differential outcomes in *Acanthamoeba* keratitis between the two locations.

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Keywords

Acanthamoeba ; Keratitis; Susceptibility; India

Introduction

Acanthamoeba spp. are free-living amoeba that exist in two life forms: a metabolically active trophozoite phase and a dormant cyst phase.[1] The trophozoite phase is readily sensitive to medical therapy while the cyst form is resistant to medications and other extreme environments. Various topical antimicrobial agents have been used to treat *Acanthamoeba* keratitis. Therapy is directed at clearing the cornea from *Acanthamoeba* cysts, though this often proves challenging, and prolonged treatment courses are the norm.

The prognosis of *Acanthamoeba* keratitis (AK) is thought to be poorer in low- and middle-income countries like India relative to high-income countries.[2–6] Variability in outcomes may be explained by the same factors that make most diseases worse in resource-limited settings, such as delays in diagnosis and treatment and lack of medicines or surgery. But it is also possible that *Acanthamoeba* susceptibility could differ based on geographic region, especially since varying resistance profiles have been reported from clinical and environmental isolates.[7–11] In this study, we compared the susceptibility of *Acanthamoeba* cysts isolated from Aravind Eye Hospital in Madurai, India and the Francis I. Proctor Foundation in San Francisco, California to determine whether susceptibility patterns were different between the two locations.

Materials and Methods

This study did not involve human subjects so ethical approval was not required.

Acanthamoeba Isolates

Acanthamoeba isolates were obtained from corneal scrapings of patients seen at the Proctor Foundation or Aravind Eye Hospital Madurai from 2008–2012. *Acanthamoeba* was originally identified on non-nutrient agar with *Escherichia coli* (*E. coli*) overlay; *Acanthamoeba* isolates were left in the original media and allowed to encyst spontaneously (i.e., the “time method” of encystment) and then stored at ambient temperature.[12] For the present study, a sample of cysts from the original petri dish was re-plated on non-nutrient agar with *E. coli* overlay, with cysts allowed to form over the course of 1 week via the “time method.” The expanded population of cysts were then transferred to tubes of normal saline, and the cyst concentration was titrated to 10^4 cysts/mL using a hemocytometer.

Antimicrobial Microdilution

Five drugs commonly used in the treatment of AK were selected for anti-amoebic susceptibility testing: the biguanide agents chlorhexidine and polyhexamethylene biguanide (PHMB), the diamidine agents hexamidine and propamidine, and the triazole agent voriconazole.[13–15] Stock solutions were made based on doses commonly used in the treatment of AK: PHMB 0.02% (200 μg/ml; compounded by Leiter’s Pharmacy),

chlorhexidine 0.02% (200 μg/ml; compounded by Leiter's Pharmacy), 1% voriconazole eye drops formulated from generic voriconazole powder (10,000 μg/ml; Sandoz), hexamidine 0.1% (1000 μg/ml; Bausch and Lomb), and propamidine 0.1% (1000 μg/ml; Sanofi). Sterile water was used to perform two-fold dilutions for each drug. Dilutions were stored at 4° C.

Minimum Cysticidal Concentration (MCC) Assay

MCC assays were performed from Fall of 2012 through Spring of 2014 using a previously described microdilution assay.[1,16,17] Clean 96-well plates (Thermo Scientific) were used for the assay. Fifty μl aliquots of each antimicrobial dilution were added to consecutive wells, followed by a 50 μl aliquot of the *Acanthamoeba* cyst suspension (which resulted in a further two-fold dilution of each antimicrobial). Two rows were reserved on each plate for positive and negative controls (i.e., *Acanthamoeba* without drug, and drug without *Acanthamoeba*, respectively). After being incubated with antimicrobial at 30° C for 48 hours, the plates were spun down at 1500 rpm for 5 minutes, and fluid was aspirated and discarded from each well. Sterile Page saline was used to wash each well in an attempt to remove any remaining antimicrobial; the fluid was aspirated and then this wash procedure was repeated 2 more times. One hundred μl of 0.5 McFarland standard *E. coli* suspension was then added to each well, and the plate was incubated at 30° C for 7 days, at which time each well was examined using an inverted microscope (Nikon) to determine trophozoite growth. The MCC for each drug was determined as the lowest drug concentration at which no trophozoites were found. The assay was run in triplicate, with the median MCC value used for analyses. All plates demonstrated trophozoite presence in the positive controls and absence in the negative control wells.

Statistical Analysis

A Wilcoxon rank-sum (Mann-Whitney U) test was performed to compare the MCC of the Indian vs. US isolates. Given the 5 comparisons, the significance level was set to $P<0.01$. All statistical analyses were performed using *R* version 4.1.0 (The R Foundation for Statistical Computing).

Results

The MCC assay was performed on *Acanthamoeba* isolates from 43 different patients (20 Aravind, 23 Proctor). No significant difference in MCC was detected between the Indian and US isolates for any drug, with broadly overlapping distributions of MCCs for each of the drugs tested (Table 1).

Discussion

In this study, we compared the MCC of PHMB, chlorhexidine, voriconazole, hexamidine, and propamidine on *Acanthamoeba* cysts isolated from patients with AK in India and the US. MCCs observed in this study were consistent with those from previous studies that have used similar methods.[1,17] We found no significant differences in MCC for any drug between the two geographic regions. Thus, this study does not provide evidence of

systematic differences in antimicrobial susceptibility between AK isolates from South India and Northern California.

Case series of AK tend to show worse visual acuity outcomes in India compared to those from high-income nations.[2–6,18] We sought to determine in this study whether *Acanthamoeba* susceptibility could play a role in the poorer outcomes in Indian AK patients. We did not find evidence of a difference in anti-amoebic susceptibility between South India and Northern California, which argues against anti-amoebic resistance as a chief driver of differential outcomes in India. Our results are supported by studies that have found similar strains of *Acanthamoeba* causing keratitis in India and in higher income countries.[19–21] It is not difficult to speculate about other reasons for differential outcomes between India and the US. Delays in diagnosis and initiation of treatment may be more common in India, and co-infection with other pathogens more likely.[22] AK infections in India are also much more likely to be due to agricultural trauma, whereas AK in higher-income countries are usually due to contact lens wear.[2,6,23] It is possible that agricultural trauma results in deeper infections or a higher inoculum, which may play a role in disease severity.

One challenge in treating acanthamoeba keratitis is the relatively poor corneal penetration of commonly used agents. For example, neither chlorhexidine nor PHMB was detectable in the anterior chambers of rabbits, even when the corneas were de-epithelialized.[24] Voriconazole has been measured at concentrations of 3.2–6.49 μg/mL in the aqueous humor after topical administration, which would not approach the MCC observed in the present study.[25,26] We are unaware of studies that have assessed the corneal penetration of the diamidine agents. Agents with better corneal penetration are needed for this difficult-to-treat infection.

Our study has limitations. Information about risk factors, clinical presentation, and outcomes was not collected as part of this laboratory study. However, it is extremely likely based on previous studies that most AK seen at Aravind would have been caused by agricultural trauma among non-contact lens-wearers, whereas most AK in California would have been observed among contact lens wearers.[3,27] *Acanthamoeba* susceptibility assays are noisy and subject to misclassification, which could bias the results toward the null and reduce the statistical power to determine a difference. The precision of MCC estimates was limited by the sample size, although it's worth noting that the present study was larger than most *Acanthamoeba* susceptibility studies.[1,28,29] We did not examine the species or genotypes of *Acanthamoeba* isolates (consistent with routine practice in most clinical microbiology laboratories), though we acknowledge that the species of clinical *Acanthamoeba* isolates may be associated with susceptibility.[21,30,31] Finally, while the isolates included in the study should be broadly representative of AK in the two study locations, the generalizability of the results to other locations is not clear.

In conclusion, we found no significant differences in the MCC for PHMB, chlorhexidine, voriconazole, hexamidine, or propamidine when comparing clinical AK isolates from South India and Northern California. This study provides no evidence to suggest that treatment choices should be different based on the geographical setting of AK infection.

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Table 1:

MCC for antiamoebic agents in Indian vs. US Isolates

Drug	Median (Interquartile range)		*p*-value*
	Aravind	Proctor	
PHMB	6.25 (5.47–12.5)	6.25 (6.25–9.375)	0.75
Chlorhexidine	6.25 (3.125–6.25)	3.125 (3.125–9.375)	0.81
Voriconazole	2500 (2500–5000)	5000 (1250–20000)	0.25
Hexamidine	15.6 (15.6–39.0625)	15.6 (15.6–31.25)	0.92
Propamidine	15.6 (7.81–15.6)	15.6 (7.81–31.25)	0.42

MCC = minimum cysticidal concentration; PHMB = polyhexamethylene biguanide

* *p*-value calculated using Wilcoxon rank sum test