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Activity of antibiotics against *Fusarium* and *Aspergillus*

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Abstract

Background/Aims—To study the susceptibility of *Fusarium* and *Aspergillus* isolated from keratitis to amoxicillin, cefazolin, chloramphenicol, moxifloxacin, tobramycin, and benzalkonium chloride (BAK).

Methods—10 isolates of *Fusarium* and 10 isolates of *Aspergillus* from cases of fungal keratitis at Aravind Eye Hospital in South India were tested using microbroth dilution for susceptibility to amoxicillin, cefazolin, chloramphenicol, moxifloxacin, tobramycin, and BAK. The minimum inhibitory concentration (MIC) median and 90th percentile were determined.

Results—BAK had the lowest MIC for both *Fusarium* and *Aspergillus*. Chloramphenicol had activity against both *Fusarium* and *Aspergillus*, while moxifloxacin and tobramycin had activity against *Fusarium* but not *Aspergillus*.

Conclusions—The susceptibility of *Fusarium* to tobramycin, moxifloxacin, chloramphenicol, and BAK and of *Aspergillus* to chloramphenicol and BAK may explain anecdotal reports of fungal ulcers that improved with antibiotic treatment alone. While some of the MICs of antibiotics and BAK are lower than the typically prescribed concentrations, they are not in the range of antifungal agents such as voriconazole, natamycin, and amphotericin B. Antibiotics may, however, have a modest effect on *Fusarium* and *Aspergillus* when used as initial treatment prior to identification of the pathologic organism.

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Keywords

keratitis; *Fusarium*; *Aspergillus*; anti-bacterial agents

INTRODUCTION

Fungal keratitis is a leading cause of ocular morbidity throughout the world, particularly in warm climates and developing countries. It is estimated that corneal ulceration and ocular trauma result in 1.5 to 2 million new cases of corneal blindness annually.[1] In certain parts of the world, such as South India, nearly half of these corneal ulcers may be secondary to fungal infection.[2] Epidemiologic studies in India have identified filamentous fungi, *Fusarium* and *Aspergillus*, as the two most common causes of fungal keratitis.[2,3] While the incidence of fungal ulcers in the United States and Europe has historically been much lower, the 2006 outbreak of *Fusarium* keratitis related to contact lens wear and the use of specific contact lens solutions has highlighted the challenges of effectively treating fungal keratitis.

Traditionally, fungal keratitis is treated medically with antifungal agents such as natamycin, amphotericin B, and voriconazole.[4,5] With failure of medical therapy, surgical interventions such as penetrating keratoplasty may be required. In the last few years, however, there have been several anecdotal cases of fungal keratitis that were initially treated with antibiotics and improved with antibiotic therapy alone. Eleven cases of the 66 patients diagnosed with *Fusarium* keratitis during the outbreak in Singapore improved with antibiotic treatment alone.[6] An additional report of 5 cases of culture-proven fungal keratitis in Florida showed significant improvement with topical fluoroquinolone therapy as initial treatment, although one case did eventually require topical natamycin treatment.[7] Furthermore, a randomized trial of corneal ulcer prevention in south India where 374 patients with corneal abrasions were randomized to either 1% chloramphenicol plus 1% clotrimazole or 1% chloramphenicol plus placebo found equivalent reduction in the development of both fungal and bacterial keratitis compared to historical controls, suggesting that antibiotic prophylaxis of corneal abrasions alone may be sufficient in preventing both bacterial and fungal infection.[8]

In vitro studies have shown activity of tobramycin against *Fusarium oxysporum*, possible activity of fourth-generation fluoroquinolones against *Fusarium* and *Candida* species, and activity of amoxicillin and erythromycin against the pectolytic and cellulolytic enzymes in *Fusarium oxysporum*.[9-11] Chodosh et al. added tobramycin solution to brain heart infusion agar prior to inoculation with one strain of *Fusarium oxysporum* isolated from a case of fungal keratitis and found that tobramycin completely inhibited growth at 450 μg/ml.[9] Ozdek et al. found inhibition of *Candida* species by moxifloxacin and gatifloxacin, and Alfonso and Miller also reported in an ARVO abstract that commercial preparations of both gatifloxacin and moxifloxacin reduced colony counts of *Fusarium oxysporum* and *Candida albicans* (Invest Ophthalmol Vis Sci 46: E-Abstract 2766, 2005).[10] However, Chai et al. found that moxifloxacin only inhibited one of ten *Fusarium* corneal isolates (Invest Ophthalmol Vis Sc. 2007 48: E-Abstract 2679, 2007). These case series and *in vitro* studies have raised the question of whether or not topical antibiotics alone may offer clinically significant antifungal activity in early fungal keratitis. In this study, we investigate the efficacy of antibiotics (amoxicillin, cefazolin, chloramphenicol, moxifloxacin, and tobramycin) and a biocidal agent (benzalkonium chloride) against *Fusarium* and *Aspergillus* species isolated from cases of fungal keratitis at a tertiary eye center in South India.

MATERIALS AND METHODS

This prospective, nonrandomized interventional study was performed in the Microbiology Department of the Aravind Eye Hospital, Madurai, India after approval by the institutional review boards of the Aravind Medical Research Foundation and the University of California San Francisco.

As previously described by Lalitha et al,[12] each patient with keratitis underwent a detailed history and clinical examination consistent with the standard of care at the Aravind Eye Hospital. Scraping of the ulcer was performed with a Kimura spatula and evaluated with Gram stain and 10% potassium hydroxide mount, then cultures were plated onto blood agar and potato dextrose agar. Fungal cultures were permitted to grow for at least 7 days at room temperature and fungal isolates were identified to the genus level, with *Aspergillus* isolates identified to the species level.

Ten fungal cultures each were randomly selected from consecutive cases of culture-proven *Fusarium* keratitis and culture-proven *Aspergillus flavus* keratitis between January 1, 2006 and December 31, 2006. Of the ten *Fusarium* isolates, all of the samples were processed. One of the *Aspergillus* isolates was contaminated by bacteria and discarded. The remaining 10 *Fusarium* isolates and 9 *Aspergillus* isolates were tested for susceptibility to 5 antibiotic agents (amoxicillin, cefazolin, chloramphenicol, moxifloxacin, and tobramycin) and 1 biocidal agent (benzalkonium chloride). For each antibiotic and biocidal agent, a range of concentrations from 1 to 4000 μg/ml was tested. Prior to susceptibility testing, each antibiotic agent was tested against a standard *Staphylococcus aureus* strain to ensure that the MIC was within the published range. One randomly selected *Fusarium* strain and one randomly selected *Aspergillus* strain were tested against amphotericin B to check for antifungal resistance.

Susceptibility was determined according to methods outlined in Clinical and Laboratory Standards Institute document M38-A.[13] The inoculum was prepared by overlaying mature slants with sterile distilled water and gently scraping the surface with a plastic pipette tip. The suspension was permitted to sit for 5 minutes to allow large particles to settle out. The inoculum was then adjusted with Mueller Hinton broth to a 0.5 McFarland standard, providing an inoculum concentration of 0.4 to 5×10^4 CFU/ml, which was verified by colony count. Mueller Hinton broth was used for the inoculum, antibiotics, and benzalkonium chloride instead of RPMI-1640 since it is a standard antibiotic test medium. Broth microdilutions were created by adding 0.1 ml of 2x concentrated drug to 0.1 ml of inoculum. Tests were incubated at 35°C for 48 hours. Tests on each fungal culture were repeated on a different date by a masked operator to reduce the effect of random variation in test results.

Since there is no standardized endpoint for the minimum inhibitory concentration (MIC) for antibiotic and biocidal agents against fungus, the MIC was defined as the lowest concentration of an antibiotic or biocidal agent that substantially inhibited growth (approximately 50% of the drug-free growth control) of the organism as detected visually at 48 hours. Given that the tests for each fungal strain were repeated twice, the geometric mean of the MIC for each antibiotic or biocide was obtained. The MIC median (MIC_{50}) and 90^{th} percentile (MIC_{90}) were determined for *Fusarium* and *Aspergillus* species (PERCENTILE function in Microsoft Excel; Microsoft Inc, Redmond, Wash).

After the MIC was determined at 48 hours, the minimum lethal concentration (MLC) was determined by plating 100 μl of the drug-free control tube, the MIC tube concentration, and each concentration above the MIC tube to a potato dextrose agar quadrant. Plates were incubated at 35°C and read at the first 24 hour interval where growth was detected in the control tube quadrant. The MLC was determined to be the lowest concentration which exhibited 5 colonies or less, equating a 95% killing.

RESULTS

The MIC_{50} and MIC_{90} for each antibiotic and biocidal agent against *Fusarium* and *Aspergillus* are displayed in Table 1. BAK had the lowest MIC for both *Fusarium* and *Aspergillus*. Chloramphenicol had activity against both *Fusarium* and *Aspergillus* at relatively high concentrations, while moxifloxacin and tobramycin had activity against *Fusarium* but not *Aspergillus*. For *Fusarium*, moxifloxacin had an MIC_{50} = 1000 μg/ml and MIC_{90} = 2000 μg/ml, while tobramycin an MIC_{50} = 500 μg/ml and MIC_{90} = 700 μg/ml. Neither amoxicillin nor cefazolin had any detectable activity against *Fusarium* or *Aspergillus* at concentrations of 4000 μg/ml and below. One hundred percent of masked replications of MICs were within one four-fold dilution.

For agent-organism combinations which had an MIC less than 4000 μg/ml, the MLC_{50} and MLC_{90} were also calculated and these are displayed in Table 2. For *Fusarium*, the MLC_{50} and MLC_{90} of tobramycin, moxifloxacin, and chloramphenicol were all >4000 μg/ml and for *Aspergillus*, the MLC_{50} and MLC_{90} of chloramphenicol were also >4000 μg/ml. The MLC_{50} and MLC_{90} for BAK against *Fusarium* and *Aspergillus* were very close to the MIC_{50} and MIC_{90}, indicating that BAK had a fungicidal rather than static effect against both *Fusarium* and *Aspergillus*.

When considering the clinical significance of the MIC_{50} and MIC_{90} for each of these antibiotic and biocidal agents, it is important to recognize that their prescription doses can vary widely for the same medication and between medications. For example, tobramycin is available at a 0.3% concentration (equivalent to 3000 μg/ml) in eyedrop form but is also given in a fortified concentration of 14 mg/ml (equivalent to 14,000 μg/ml). Commonly prescribed dosages of these medications are given in Table 3.

DISCUSSION

Of the antibiotic agents, chloramphenicol had an effect against both *Fusarium* and *Aspergillus* while tobramycin and moxifloxacin had an effect only against *Fusarium*. Though a few of the antibiotics tested in this study showed efficacy against *Fusarium* and *Aspergillus*, these MICs are much higher than comparable values for antifungal agents such as amphotericin B, natamycin, and voriconazole. For example, a recent report from our group that examined similar isolates found that the MIC_{50} for amphotericin B against *Fusarium* was 2 μg/ml and the MIC_{50} for voriconazole against *Fusarium* was 0.25 μg/ml.[12] In the current study, quality control tests with amphotericin B found an MIC of 0.5 μg/ml against two randomly selected *Fusarium* and *Aspergillus* strains. Therefore, antifungal agents remain imperative for first-line treatment of fungal keratitis. The results of this study, however, may explain anecdotal reports of fungal keratitis that improved after initial treatment with antibiotics. In particular, tobramycin is often given in a fortified concentration of 14 mg/ml (14,000 μg/ml), which is 30-fold higher than the MIC_{50} for tobramycin against *Fusarium* (500 μg/ml). This MIC value is also consistent with Chodosh et al's report that tobramycin completely inhibited growth of one strain of *Fusarium oxysporum* at 450 μg/ml.[8] Even considering the effect of tear film dilution, tobramycin would likely have some measurable activity against *Fusarium* keratitis. Of note, any synergy offered by an antibiotic in conjunction with an antifungal agent would not have been identified in these experiments.

The molecular basis for the efficacy of antibiotics against fungus is not completely understood. Fluoroquinolones like moxifloxacin and gatifloxacin work against type II topoisomerase DNA gyrase and topoisomerase IV, thus the presence of DNA topoisomerases I and II in yeast cells has been proposed as a mechanism for these agents' antifungal activity.[7] Aminoglycosides are thought to make fungal cell walls and membranes more permeable to ionic shifts, perhaps

explaining the antifungal effect of tobramycin in this study.[14] Chloramphenicol has been shown to have some *in vitro* effect against the polygalacturonase and pectinmethylgalacturonase enzymes of *Fusarium oxysporum*.[11]

The mechanisms of action of biocidal agents like benzalkonium chloride are arguably less well elucidated than those of antibiotic agents. Benzalkonium chloride is one of the quarternary ammonium compounds (QACs), membrane-active agents which primarily target the cytoplasmic (inner) membrane of bacteria of the plasma membrane of yeasts.[15] The following sequence of events has been proposed in micro-organisms exposed to QACs: adsorption and penetration of the agent into the cell wall, reaction with the cytoplasmic membrane followed by membrane disorganization, leakage of low-molecular-weight material, degradation of proteins and nucleic acids, and wall lysis caused by autolytic enzymes.[15] *In vitro* testing of the fungicidal activity of benzalkonium chloride against *Aspergillus fumigatus* showed biocidal activity in less than 5 minutes of contact time, defined as a 10^4 or more reduction in viability of *A. fumigatus* strains.[16]

The finding of relatively low MIC and MLC values for BAK against both *Fusarium* and *Aspergillus* raises the question of whether the addition of benzalkonium chloride to antifungal agents for treatment or prophylaxis may be beneficial for its fungicidal activity in addition to its preservative qualities. Ozdek et al found that Zymar® (0.3% gatifloxacin + 0.005% BAK, Allergan Inc., Irvine, CA) had greater in vitro activity than Vigamox® (0.5% moxifloxacin, Alcon Laboratories, Fort Worth, TX) against *Candida* isolated from ocular infections, and they postulated that this effect was due to the presence of BAK in Zymar.[10] Similarly, Alfonso and Miller found that Zymar® (0.3% gatifloxacin + 0.005% BAK, Allergan Inc., Irvine, CA) reduced the colony count of both *Fusarium oxysporum* and *Candida albicans* by 99.9% at 1,4, and 24 hours, while Vigamox® (0.5% moxifloxacin, Alcon Laboratories, Fort Worth, TX) had similar results for *Fusarium oxysporum* but not *Candida albicans* (Invest Ophthalmol Vis Sci 46: E-Abstract 2766, 2005). However, it should be noted that the MIC_{50} of BAK against filamentous fungus is only 1 to 6-fold lower than the concentration typically found in ophthalmic preparations, and the use of higher concentrations or more frequent dosing of BAK is limited by toxicity.

Although several authors have investigated the *in vitro* effect of fluoroquinolones and tobramycin on *Fusarium*, this is the first study using the CLSI broth microdilution antifungal susceptibility testing to determine the MICs of antibiotics versus *Fusarium* and *Aspergillus*.[13] While *in vitro* testing must eventually be studied in conjunction with *in vivo* clinical results, the correlation of *in vitro* fungal drug-susceptibility testing with *in vivo* therapy and clinical outcomes has gained credibility in the non-ophthalmic literature.[17] Similar to susceptibility testing for bacterial disease, approximately 90% of infections with susceptible isolates will respond to therapy, while only 60% of infections due to resistant isolates respond to therapy.[17] Further studies would need to be conducted to assess whether tobramycin, moxifloxacin, chloramphenicol, and BAK might have a clinically relevant additive, antagonistic, or synergistic *in vivo* effect with antifungal agents.

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Table 1

MIC_{50} and MIC_{90} results against *Aspergillus* and *Fusarium*

	Amoxicillin		Cefazolin		Chloram-phenicol		Moxifloxacin		Tobramycin		Benzalkonium Chloride	
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
***Fusarium* spp**	>4000	>4000	>4000	>4000	1000	2000	1000	2000	500	700	32	64
Aspergillus flavus	>4000	>4000	>4000	>4000	2000	4000	>4000	>4000	>4000	>4000	16	16

Table 2

MLC_{50} and MLC_{90} results against *Aspergillus* and *Fusarium*

	Chloram-phenicol		Moxifloxacin		Tobramycin		Benzalkonium Chloride	
	MLC_{50} (μg/ml)	MLC_{90} (μg/ml)	MLC_{50} (μg/ml)	MLC_{90} (μg/ml)	MLC_{50} (μg/ml)	MLC_{90} (μg/ml)	MLC_{50} (μg/ml)	MLC_{90} (μg/ml)
***Fusarium* spp**	>4000	>4000	>4000	>4000	>4000	>4000	64	64
Aspergillus flavus	>4000	>4000	N/A	N/A	N/A	N/A	16	32

Table 3

Commonly prescribed dosages of antibiotics and BAK

Chloramphenicol:	1% ointment = 10,000 μg/ml
	(Chloromycetin®, Parke-Davis, Morris Plains, NJ)
Moxifloxacin:	0.5% eyedrops = 5000 μg/ml
	(Vigamox®, Alcon Laboratories, Fort Worth, TX)
Tobramycin:	0.3% eyedrops = 3000 μg/ml
	(Tobrex®, Alcon Laboratories, Fort Worth, TX)
	14 mg/ml fortified eyedrops = 14,000 μg/ml
	(compounded by hospital pharmacies)
Benzalkonium Chloride:	0.004% in Polytrim®, Allergan Inc., Irvine, CA = 40 μg/ml
	0.005% in Zymar®, Allergan Inc., Irvine, CA = 50 μg/ml
	0.006% in Ciloxan®, Alcon Laboratories, Fort Worth, TX = 60 μg/ml
	0.01% in Tobradex®, Alcon Laboratories, Fort Worth, TX = 100 μg/ml
	0.02% in Xalatan®, Pfizer, NY, NY = 200 μg/ml