Antimicrobial Susceptibility of Fusarium, Aspergillus, and Other Filamentous Fungi Isolated From Keratitis

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Objective: To characterize the susceptibility of filamentous fungi isolated from keratitis to amphotericin B, natamycin, caspofungin acetate, itraconazole, voriconazole, and posaconazole.

Methods: Ninety isolates from fungal keratitis cases at Aravind Eye Hospital in South India were tested using macrobroth dilution for susceptibility to amphotericin B, natamycin, caspofungin acetate, itraconazole, voriconazole, and posaconazole. The minimum inhibitory concentration (MIC) median and 90th percentile were determined.

Results: The 90 isolates included 41 Aspergillus species, 38 Fusarium species, and 11 others. The triazoles and caspofungin had the lowest MICs against Aspergillus species; voriconazole, amphotericin B, and posaconazole had the lowest MICs against Fusarium species, and none of the Fusarium species were inhibited by itraconazole or caspofungin. Amphotericin B had significantly lower MICs compared with natamycin, but after correcting for the typical prescription dose, natamycin was superior.

Conclusion: No single agent was universally most effective, but voriconazole and other triazoles demonstrated the broadest spectrum. Itraconazole and caspofungin were not effective against Fusarium species.

Clinical Relevance: Fungal ulcers are commonly treated empirically; drugs are typically selected without regard to susceptibility data. The nonocular infectious disease literature suggests modern fungal susceptibility methods are clinically relevant, but ocular studies are limited. Our results suggest antifungal therapy might be tailored to individual organisms.

Arch Ophthalmol. 2007;125:789-793

OVER THE PAST 4 DECADES, there has been an increase in the percentage of infectious keratitis caused by fungal infection.1,2 Keratitis is a leading cause of monocular blindness worldwide,3,4 and it is estimated that more than half of corneal ulcers in some areas of the world are due to fungus.5 Filamentary fungal ulcers are thought to have a particularly poor prognosis.6 Only 1 antifungal agent, natamycin, is approved by the US Food and Drug Administration for topical ophthalmic use, although several others have been studied in vitro and in animal models or used clinically in compounded formulations.7-11 Despite the advent of improved laboratory diagnosis and increasing evidence that fungal susceptibility testing provides clinically relevant information, the treatment of fungal ulcers is still most commonly empirical.12,13 The absence of laboratory studies results in a gap in the ophthalmic literature regarding the potential application of emerging antifungal agents to fungal keratitis. Linares et al14 determined the susceptibility of 244 fungal isolates, but only 14 isolates were Fusarium species, and these were from nonocular disease. Marangon et al15 reviewed 419 cases of fungal keratitis but only tested the susceptibility of 9 Fusarium species and 4 Aspergillus species. Neither of these studies determined the susceptibility of isolates to natamycin, posaconazole, or caspofungin.

Classes of antifungal medications used for filamentary fungal keratitis include the polyenes, triazoles, and echinocandins.12 Natamycin, a polyene, has long been considered the mainstay of treatment for filamentous fungal keratitis. However, for systemic diseases such as pulmonary aspergillosis, newer triazoles such as voriconazole are the recommended therapy.16 In the past, techniques for performing antifungal susceptibility testing have been difficult to perform and standardize. Consequently, many of the new antifungal agents...
RESULTS

The 90 processed isolates included 41 *Aspergillus* species, 38 *Fusarium* species, and 11 others (Table 1), allowing for MIC₅₀ and MIC₉₀ estimation of these 2 principle organisms (Table 2). The MICs ranged markedly among antifungal agents and organisms. The triazoles performed best against *Aspergillus* species (Figure 1A), with posaconazole having significantly lower MICs than the second most effective agent, voriconazole (P<.001). Voriconazole and amphotericin B had the lowest MICs vs *Fusarium* species, and they were not significantly different from each other (P = .21) (Figure 1B). None of the 38 *Fusarium* species were inhibited at even the highest tested concentration of itraconazole (8 µg/mL) or caspofungin (16 µg/mL). Compared with natamycin, amphotericin B had significantly lower MICs vs *Aspergillus* species (P<.001) and vs *Fusarium* species (P<.001).

Each antifungal has a typical prescription dose that can differ markedly from other antifungals. For example, amphotericin B is regularly given at 0.15%, whereas natamycin is usually given at 5%. Therefore, 1 drop of natamycin has 30 times the amount of drug than 1 drop of amphotericin B. Figure 2 normalizes the MIC₅₀ relative to the typical prescription dose. Compared with amphotericin B, natamycin had significantly lower MICs against *Aspergillus* species (P = .006) and against *Fusarium* species (P<.001). While amphotericin B had nearly the same absolute MICs as voriconazole vs *Fusarium* spe-

### Table 1. Distribution of Fungal Isolates

<table>
<thead>
<tr>
<th>Organism Isolated</th>
<th>Isolates Collected (n = 98)</th>
<th>Isolates Processed (n = 90)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus flavus</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Fusarium species</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>Curvularia species</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Bipolaris species</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Cladosporium species</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Acremonium species</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Botryodiplodia species</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Exserohilum species</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Trichoderma species</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Unidentified hyaline fungus</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

*Of the 98 consecutive cases of culture-positive fungal keratitis seen at Aravind Eye Hospital between March and July 2004, 8 isolates were contaminated by bacteria and could not be processed for susceptibility testing against the 6 antifungals.

This prospective, nonrandomized interventional study was performed in the Cornea Department of 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.

Fungal cultures were isolated from 98 consecutive cases of culture-proven fungal keratitis seen at the Aravind Eye Hospital cornea clinic between March and July 2004. Consistent with the standard of care at Aravind, each patient with keratitis gave a clinical history and underwent a detailed clinical examination using a slitlamp biomicroscope to measure the size and depth of ulceration. Scraping of the corneal ulcer was performed under magnification using a Kimura spatula, and the material obtained was evaluated with a gram stain and 10% potassium hydroxide mount. Subsequent scrapings were plated onto blood agar and potato dextrose agar and permitted to grow at room temperature for at least 7 days prior to testing. Any positive fungal isolate was identified to the genus level, with *Aspergillus* species identified to the species level.

Of the 98 isolates, 8 were not processed because of bacterial contamination. The remaining 90 isolates were tested for susceptibility to 6 antifungal agents: amphotericin B, natamycin, caspofungin acetate, itraconazole, voriconazole, and posaconazole. Susceptibility was determined according to methods outlined in Clinical and Laboratory Standards Institute documents M38-A and M27-A.²⁰,²⁸ The inoculum was prepared by overlaying mature slants with sterile distilled water and gently scraping the surface with a wooden applicator stick. The suspension was then adjusted spectrophotometrically to the correct optical density for each species as outlined in M38-A, providing an inoculum concentration of 2.5–5 x 10⁴ conidia/mL, which was verified by colony count.

Broth macrodilutions were created by adding 0.1 mL of 10 x concentrated drug to 0.9 mL of inoculum. The test medium was RPMI-1640 for the azoles and antibiotic medium 3 for the polyenes and caspofungin.²⁹-³³ Tests were incubated at 35°C until growth was visible in the drug-free control tube. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that exhibited a 100% visual reduction in turbidity when compared with the control tube for the polyenes at 48 hours.³⁰ An 80% reduction in turbidity was considered the endpoint for the azoles.³⁰ The minimum effective concentration, or the lowest concentration that induces aberrant growth, was used to determine the cutoff for caspofungin.³⁴ The MIC median (MIC₅₀) and 90th percentile (MIC₉₀) were determined for *Fusarium* species, *Aspergillus* species, and all 90 isolates together (PERCENTILE function in Microsoft Excel; Microsoft Inc, Redmond, Wash). The MICs of antifungals were compared using a Wilcoxon signed rank test (Stata 7.0; StataCorp, College Station, Tex).

The relative MIC₅₀ is included alongside the absolute MIC₅₀. The relative MIC₅₀ normalizes the discrepancies between the typical prescription doses of the 6 antifungal drugs, which range considerably from 0.15% to 5%. The relative MIC₅₀ (no units) is calculated by dividing the absolute MIC₅₀ (micrograms per milliliter) by the typical prescription dose (weight per volume percentage or grams per 100 mL). Although the relative MIC₅₀ does not account for the bioavailability or penetration of topical drugs, it does take into account that, for example, each drop of natamycin administered has 30-fold the drug concentration compared with amphotericin B, and this approach may therefore provide some additional information.
A comparison of antifungal susceptibilities for 90 fungal isolates is shown in Table 2. Voriconazole had significantly lower MICs for Aspergillus species than itraconazole or natamycin (P =.21). Caspofungin acetate and posaconazole had significantly lower MICs for Fusarium species than itraconazole (P <.001). When including all fungal isolates, voriconazole and posaconazole offered the lowest relative MICs.

**Table 2. MIC50 and MIC90 of Fungal Isolates**

<table>
<thead>
<tr>
<th>Species</th>
<th>MIC50 (µg/mL)</th>
<th>MIC90 (µg/mL)</th>
<th>MIC50 (µg/mL)</th>
<th>MIC90 (µg/mL)</th>
<th>MIC50 (µg/mL)</th>
<th>MIC90 (µg/mL)</th>
<th>MIC50 (µg/mL)</th>
<th>MIC90 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus species</em></td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>32</td>
<td>&gt;32</td>
<td>0.5</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td><em>Fusarium species</em></td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>All 90 isolates (Aspergillus species, Fusarium species, and others)</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;16</td>
<td>0.5</td>
<td>&gt;16</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Abbreviations: MIC50, minimum inhibitory concentration median; MIC90, minimum inhibitory concentration 90th percentile.

In this study, we investigated the in vitro efficacy of traditional and newer antifungals against isolates from fungal keratitis cases using macrodilution assays that represent the current gold standard. As is the case for antibiotics and bacteria, no single antifungal was most effective for all fungi. Posaconazole had the lowest MIC90 against *Aspergillus* species (n=41), closely followed by itraconazole, caspofungin, and voriconazole. Amphotericin B and voriconazole had the lowest MIC90 vs *Fusarium* species (n=38). The results for *Aspergillus* species are consistent with the nonophthalmic literature, where the newer triazoles are considered the treatment of choice for systemic aspergillosis. Both caspofungin and itraconazole were reasonably effective against *Aspergillus* species but were completely ineffective against *Fusarium* species. For itraconazole, which is available commercially as a topical agent in India, these data are consistent with clinical studies that reported itraconazole was effective against *Fusarium* species at the highest concentration tested (8 µg/mL), and therefore also has an indeterminate MIC90, although the MIC90 can be extrapolated to be slightly greater than 8 µg/mL. Itraconazole (ITRA) and caspofungin acetate (CAS) both failed to inhibit growth of *Fusarium* species at every concentration tested and are represented as horizontal lines that span the respective concentrations tested. AMB indicates amphotericin B; VOR, voriconazole. AMB and NAT are polyenes, CAS is an echinocandin, and ITRA, VOR, and POS are triazoles.

**Figure 1.** The percentage of isolates inhibited at various concentrations of antifungal agent. A, *Aspergillus* species (n=41). B, *Fusarium* species (n=38). Gray lines represent the threshold for the minimum inhibitory concentration median (MIC50) and 90th percentile (MIC90). Natamycin (NAT) inhibited only 76% of *Aspergillus* at the highest concentration tested (32 µg/mL), and therefore, the MIC90 is indeterminate. Posaconazole (POS) inhibited only 89.5% of *Fusarium* species at the highest concentration tested (8 µg/mL) and therefore also has an indeterminate MIC90, although the MIC90 can be extrapolated to be slightly greater than 8 µg/mL. Itraconazole (ITRA) and caspofungin acetate (CAS) both failed to inhibit growth of *Fusarium* species at every concentration tested and are represented as horizontal lines that span the respective concentrations tested. AMB indicates amphotericin B; VOR, voriconazole. AMB and NAT are polyenes, CAS is an echinocandin, and ITRA, VOR, and POS are triazoles.

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The effectiveness of the antifungal agent is greatly affected by the substantially different concentrations of drug achieved locally. In practice, the topical antifungal medications are given in different concentrations and this is not taken into account when comparing absolute MIC values. For example, natamycin is commercially available in a 5% concentration, while amphotericin B is typically prescribed at a concentration of only 0.15% because of toxicity. After correction for the available dose, natamycin has lower relative MICs than amphotericin B for both Fusarium species and Aspergillus species. The adjusted MIC reveals that if penetration is not an issue, natamycin may be as effective as voriconazole for fungal species and Aspergillus species, but after adjusting the MIC for the typical prescription dose, AMB had the poorest relative MIC.

Although susceptibility testing is used far more frequently for bacterial than fungal disease, fungal susceptibility testing is gaining credibility in the literature. Bench marks and in vivo clinical results will be important in the future management of fungal keratitis.

Submitted for Publication: September 18, 2006; final revision received December 6, 2006; accepted December 19, 2006.

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Financial Disclosure: None reported.

Previous Presentation: This work was presented in part at the Association for Research in Vision and Ophthalmology Annual Meeting; May 3, 2005; Fort Lauderdale, Fla.

Funding/Support: This study was supported in part by an unrestricted grant from That Man May See, Inc, San Francisco, Calif; an unrestricted grant from Research to Prevent Blindness, New York, NY; the UCSF School of Medicine Dean’s Office, San Francisco; and grant U10 EY015114 from the National Institutes of Health.

Role of the Sponsor: The funding sources did not influence the design or conduct of the study; the collection, management, analysis, or interpretation of the data; or the preparation, review, or approval of the manuscript.

REFERENCES


