In Vitro Natamycin Susceptibility of Ocular Isolates of *Fusarium* and *Aspergillus* Species: Comparison of Commercially Formulated Natamycin Eye Drops to Pharmaceutical-Grade Powder[▽]

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The Clinical and Laboratory Standards Institute susceptibility method prohibits the use of pharmacy preparations, but obtaining pure powders is difficult. The activity of natamycin against isolates of *Aspergillus* and *Fusarium* species isolated from keratitis was assessed by using both powder and pharmacy eye drop preparations. Eye drop preparations may be a viable option for testing natamycin activity.

Keratitis is a leading cause of monocular blindness worldwide (11, 12, 14, 15), and recent reports suggest that there has been a steady increase in the percentage of infectious keratitis caused by fungus (1, 12, 17). *Fusarium* and *Aspergillus* species are the most common fungal isolates associated with this infection (1, 17). Classes of antifungals used for treatment of fungal keratitis include the polyenes, triazoles, and echinocandins (13). Natamycin (NAT), a tetraene polyene, has long been considered the mainstay of treatment for filamentous fungal keratitis and is the only available antifungal medication that has been approved for this indication by the U.S. Food and Drug Administration. Although previous reports regarding the efficacy of NAT in fungal keratitis are available (4–6), there are few studies that describe the in vitro activity of NAT against the common ocular isolates (2, 7, 8, 9).

The Clinical Laboratory and Standards Institute guidelines stress the use of pharmaceutical-grade powder solely for susceptibility testing. This is due to unknown purity and potency values and additives that may impact results (3). Pharmaceutical-grade powder is very difficult to obtain, especially for drugs that are not frequently used, such as NAT. We studied the in vitro activity of NAT against *Fusarium* and *Aspergillus* species isolated from cases of corneal ulcers seen at the Aravind Eye Hospital, Madurai, India, and compared the MICs obtained by using both pharmaceutical-grade NAT powder (NAT-P) and commercially available NAT eye drops (NAT-D).

A total of 100 fungal isolates recovered from clinical cases of corneal ulcer were evaluated in the present study. The fungal isolates included 41 *Fusarium*, 32 *Aspergillus flavus*, 18 *Aspergillus fumigatus*, 5 *Aspergillus terreus*, and 4 *Aspergillus niger* isolates. The reference strain of *A. flavus* ATCC 204304 was included and was tested with both formulations in each series of assays. Antifungal susceptibility testing was performed exactly as outlined in CLSI M38-A. Pharmaceutical-grade NAT

was obtained from Alcon Laboratories (Ft. Worth, TX). A 5% NAT suspension of topical eye drops was purchased from Sun Pharmaceutical, Ltd. (Mumbai, India). Amphotericin B was used to assess quality control against *A. flavus* ATCC 204304 strains. Inocula were prepared without the use of Tween 20. NAT-P was weighed and dissolved in dimethyl sulfoxide. Stock solutions were stored frozen at −70°C until needed. Drug dilution tubes were prepared from a 3,200-μg/ml stock and diluted 1:2.5 with water to achieve a top concentration of 1,280 μg/ml. For NAT-D, 1 ml of 5% NAT suspension was mixed with 9 ml of 100% dimethyl sulfoxide (5,000 μg/ml). One part of this suspension was mixed with three parts of sterile water to give a concentration of 1,280 μg/ml. Final drug concentrations ranged from 128 to 0.25 μg/ml.

The MIC was defined as the lowest drug concentration that completely inhibited visual growth. NAT-P and NAT-D were tested simultaneously in the same plate for all of the isolates. The reference strain was tested in the same manner as the clinical isolates to verify the reproducibility for each run. The MICs of NAT-P and NAT-D were compared by using a Mann-Whitney test, and a P value of >0.05 was taken to mean that there was no significant difference found between groups. A P value of <0.05 meant a significant difference was found between groups.

The results of the MIC testing are given in Table 1. Comparison of the MICs between NAT-P and NAT-D showed perfect agreement, with 92.6% for *Fusarium* spp. (38 of 41 isolates) and 71.9% for *A. flavus* (23 of 32 isolates). Overall, for 79% of the isolates the NAT MICs were identical regardless of the drug formulation. Considering the allowable ±2 dilutions, there was only a one-dilution variation for 21% of the remaining isolates, bringing the overall agreement to 100% (Table 2).

Interestingly, susceptibility to NAT varied by species when the results for the aspergilli were examined. The NAT MICs for *A. flavus* were higher than for the other species (i.e., a NAT MIC_{90} of 64 μg/ml compared to a NAT MIC_{90} of 4 μg/ml for *A. fumigatus*). For other species, there were too few isolates to calculate the MIC_{90}.

The present study shows that the NAT had good activity against both *Fusarium* and *Aspergillus* spp., with slightly higher NAT MICs for *Aspergillus* spp. To date, susceptibility break-

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TABLE 1. MICs of NAT-P and commercially available NAT-D for 100 corneal fungal isolates

Organism	No. of isolates tested ($n = 100$)	MIC$_{50}$ (μg/ml)		MIC$_{90}$ (μg/ml)		Range (μg/ml)		P
		NAT-P	NAT-D	NAT-P	NAT-D	NAT-P	NAT-D	
Fusarium species	41	4	4	4	4	2–8	2–8	0.534
Aspergillus flavus	32	32	32	64	64	8–64	16–64	0.195
Aspergillus fumigatus	18	4	2	4	4	1–4	1–4	0.066
Aspergillus terreus	5					4–16	4–16	1.0
Aspergillus niger	4					1–4	2–4	0.343

points have not been established for NAT, but the MICs obtained here are likely within the achievable levels obtained in the eye during standard therapy. For discussion purposes, the desirable target value of ≤16 μg/ml is used to represent susceptibility. Interestingly, the MICs obtained from both the pharmaceutical-grade powder and the eye drops were comparable.

In a recent study from China on the pattern of ocular fungal isolates, it was found that *Fusarium* was the predominant pathogen and that 93% of the isolates were sensitive to NAT, whereas 92% of the *Aspergillus* isolates were sensitive to itraconazole (16, 17).

Lalitha et al., examining the susceptibility of filamentous fungi isolated from keratitis to amphotericin B, NAT, caspofungin acetate, itraconazole, voriconazole, and posaconazole, found that triazoles and caspofungin had the lowest MICs against *Aspergillus* species; that voriconazole, amphotericin B, and posaconazole had the lowest MICs against *Fusarium* species; and that none of the *Fusarium* species were inhibited by itraconazole or caspofungin. The MICs of amphotericin B were significantly lower than those of NAT, but after correction for the typical prescription dose, NAT was found to be more effective (8). It is important that the in vitro studies be correlated with the clinical outcome. Such data are limited in cases of fungal keratitis, whereas in cases of bacterial keratitis the clinical outcomes have been correlated with the sensitivities of various antibiotics, and patients in whom the antibiotic MICs were higher demonstrated a poorer outcome.

One of the main deterrents in evaluating antifungal susceptibilities is the nonavailability of a pharmaceutical grade of the

drug. Another of our other aims was to determine whether the MICs obtained with the pharmaceutical powder and the eye drops could produce comparable results. We found that the results were statistically comparable, leading us to believe that the option of using a commercial preparation of the antifungal agent might be an alternative to consider in evaluating antifungal susceptibility in situations where the pure form of the drug is not be available. Further studies with NAT-D from other manufacturers are required. In addition, similar studies with other antifungals should be performed.

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TABLE 2. Comparison of MICs of NAT-P and commercially available NAT-D

Organism	No. of isolates tested	MIC results (no. of isolates [%])	
		No. identical	No. with one dilution variation
Fusarium species	41	38 (92.6)	3 (7.3)
Aspergillus flavus	32	23 (71.9)	9 (28.1)
Aspergillus fumigatus	18	12 (66.7)	6 (33.3)
Aspergillus terreus	5	5 (100)	0
Aspergillus niger	4	1 (25)	3 (75)
Total	100	79 (79)	21 (21)