In-vitro susceptibility of Natamycin against ocular isolates of *Fusarium* and *Aspergillus* species: A comparison of the commercially formulated natamycin eye drops to pharmaceutical grade powder

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The CLSI susceptibility method prohibits pharmacy preparation use but obtaining pure powders is difficult. Natamycin activity 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 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, 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 Food and Drug Administration of the USA. Although previous reports regarding the efficacy of natamycin in fungal keratitis \[4,5,6\] are available, there are few studies which describe the in-vitro activity of natamycin against the common ocular isolates \[7,8,9\].

The Clinical Laboratory and Standards Institute (CLSI) 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 MIC values obtained by using both pharmaceutical grade Natamycin powder (NAT-P) and commercially available Natamycin eye drops (NAT-D).

A total of 100 fungi recovered from clinical cases of corneal ulcer were evaluated in this study. The fungal isolates included 41 *Fusarium* spp., 32 *Aspergillus flavus*, 18 *Aspergillus fumigatus*, 5 *Aspergillus terreus* and 4 *Aspergillus niger*. The reference strain of *Aspergillus flavus* ATCC 204304 was included and was tested with both formulations.
in each series of assays. Antifungal susceptibility testing was performed exactly according to the method outlined in CLSI M38-A. Natamycin pharmaceutical grade 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 *Aspergillus flavus* ATCC 204304 strains. Inocula were prepared without the use of Tween 20. NAT-P was weighed and dissolved in DMSO. Stock solutions were stored frozen at -70°C until needed. Drug dilution tubes were prepared from 3200 µg/ml stock and diluted 1:2.5 with water to achieve a top concentration of 1280µg/ml. For NAT-D, 1ml of 5% natamycin suspension was mixed with 9ml of 100% DMSO (5000 µg/ml). One part of this suspension was mixed with 3 part of sterile water to give a concentration of 1280 µg/ml. Final drug concentrations ranged from 128 to 0.25 µg/ml.

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

Results of MIC testing are given in Table 1. The comparison of MIC values between NAT-P and NAT-D showed perfect agreement with 92.6% for *Fusarium* spp. (38/41) and 71.9% for *Aspergillus flavus* (23/32). Overall 79% isolates gave identical MIC values regardless of drug formulation. Considering the allowable +/- 2 dilutions,
21% of the remaining isolates showed only a one-dilution variation bringing the overall agreement to 100% (Table 2).

Interestingly, susceptibility to NAT varied by species when reviewing the results for the aspergilli. *Aspergillus flavus* had higher MICs than other species with the MIC$_{90}$ of 64 µg/ml as compared to *A. fumigatus* with a MIC$_{90}$ of 4 µg/ml. Other species contained too few isolates to calculate the MIC$_{90}$.

This study showed that the NAT had good activity against both *Fusarium* and *Aspergillus* spp. with *Aspergillus* spp. having slightly higher MICs. To date, susceptibility breakpoints have not been established for NAT but the MICs obtained 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 predominate pathogen and 93% of the isolates were sensitive to natamycin while 92% of *Aspergillus* was sensitive to itraconazole. [17] A study by Lalitha et al in which the susceptibility of filamentous fungi isolated from keratitis to amphotericin B, natamycin, caspofungin acetate, itraconazole, voriconazole, and posaconazole was studied found that 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 [8].

It is important that the in vitro studies be correlated with the clinical outcome. Such data is limited in cases of fungal keratitis whereas in cases of bacterial keratitis the clinical outcomes have been correlated to sensitivities of various antibiotics where patients with higher MIC’s had a poorer outcome.

One of the main deterrents in doing antifungal susceptibilities is the non-availability of the pharmaceutical grade of the drug. Another of our other aims was to determine if the MICs obtained by the pharmaceutical powder and the eye drops could produce comparable results. We found that the results were statically comparable leading us to believe that the option of using commercial preparation of the antifungal agent might be an alternative to consider in doing antifungal susceptibility in situations where the pure form of the drug may not be available. Further studies with NAT-D from other manufacturers are required. In addition, similar studies with other antifungals should be performed.
References:


Table 1

MIC values for Natamycin pharmaceutical powder (NAT-P) and commercially available Natamycin eye drops (NAT-D) against 100 corneal fungal isolates

<table>
<thead>
<tr>
<th>ISOLATE</th>
<th>No. Tested (N – 100)</th>
<th>MIC_{50}</th>
<th>MIC_{90}</th>
<th>Range</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NAT-P</td>
<td>NAT-D</td>
<td>NAT-P</td>
<td>NAT-D</td>
</tr>
<tr>
<td>Fusarium species</td>
<td>41</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>18</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>A. terreus</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. niger</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>
Comparison of MIC results of Natamycin pharmaceutical powder (NPP) and commercially available Natamycin eye drops (NED)

<table>
<thead>
<tr>
<th>ISOLATE</th>
<th>Number Tested</th>
<th>Identical results</th>
<th>One dilution variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium species</td>
<td>41</td>
<td>38 (92.6%)</td>
<td>3 (7.3%)</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>32</td>
<td>23 (71.9%)</td>
<td>9 (28.1%)</td>
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<tr>
<td>Aspergillus fumigatus</td>
<td>18</td>
<td>12 (66.7%)</td>
<td>6 (33.3%)</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>5</td>
<td>5 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>4</td>
<td>1 (25%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
<td><strong>79 (79%)</strong></td>
<td><strong>21 (21%)</strong></td>
</tr>
</tbody>
</table>