PHILIPPINE JOURNAL OF
Ophthalmology
VOL. 29 • NO. 1 March 2004

ORIGINAL ARTICLE

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In vitro evaluation of natamycin 5% suspension against *Aspergillus flavus, Fusarium solani,* and *Candida parasilopsis*

ABSTRACT

Objective

This study compared the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of two available brands of natamycin 5% suspension (Natacyn and Elmycin) against three ocular fungi *(Aspergillus flavus, Candida parasilopsis, Fusarium solani).*

Methods

Antifungal susceptibility testing by broth microdilution was performed. The MIC and MFC of both brands were determined and paired *t*-tests were compared.

Results

Results of MIC and MFC of Elmycin and Natacyn against *Aspergillus flavus* showed no significant difference ($p = 0.05$). The same values were obtained for *Fusarium solani* and *Candida parasilopsis,* showing no difference in their MIC and MFC.

Conclusion

Elmycin and Natacyn have similar MIC and MFC against *Aspergillus flavus, Fusarium solani,* and *Candida parasilopsis* as determined by *in vitro* tube dilution technique. Elmycin may be used as an alternative agent against these organisms in fungal keratitis.

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Presented at the Philippine Academy of Ophthalmology Annual Convention, November 2003.

The authors have no proprietary or financial interest in any product described in this study.

PHILIPP J OPHTHALMOL 2004; 29 (1):26-28

THE INCIDENCE of fungal keratitis has increased over the past 30 years. In the United States, it ranges from 2% of all keratitis cases in New York to 35% in Florida.[1] *Fusarium* is the most common cause of fungal corneal infection in southern states (45-76% of fungal keratitis) while *Candida* and *Aspergillus* are more common in northern states.[2] In South Florida, *Fusarium oxysporium* was the most common isolate (37%) followed by *F. solani* (24%), *Candida, Curvularia,* and *Aspergillus.*[2]

But elsewhere in the world, *Aspergillus* is the most common isolate. In India, it accounts for 2-64% of cases followed by *Fusarium* (6-32%) and *Pennicilium sp.* (2-29%).[1] In the Philippines, a review by Valenton of 3,256 microbial keratitis cases treated at the Philippine General Hospital from 1972 to 1996 reported 349 laboratory confirmed cases of fungal keratitis. Of these, 105 were caused by *Fusarium sp.* and 26 by *Aspergillus sp.*[3]

The current treatment protocol for fungal keratitis recommends 0.1% amphotericin B or 5% natamycin as first-line antifungal agents. Also used are polyene antibiotics (nystatin, amphotericin B, natamycin); pyrimidine analogs (flucytosine); imidazoles (clortrimazole, miconazole, ketoconazole); triazoles (fluconazole, itraconazole); silver sulfadiazine; chemotherapeutic agents; and corticosteroids.[4, 5, 6]

Valenton studied 309 fungal keratitis patients treated with topical antimicrobial after superficial keratectomy of ulcer infiltrate. The response rate was 33% for patients treated with plain topical antibiotics, 55% for those treated with topical amphotericin B (Fungizone, Bristol-Myers Squibb, New York, NY, USA), 54% for those treated with topical natamycin (Natacyn, Alcon Laboratories, Fort Worth, TX, USA) applied 6 times daily, and 33% for those treated with miconazole suspension (GynoDaktarin ovule 40mg in 5cc liquifilm ophthalmic solution, Janssen-Cilag, Mexico).[3]

Natamycin is the only commercially available topical antifungal. It is a polyene macrolide produced by Streptomyces natalensis, which is structurally related to amphotericin B and nystatin. *In vitro*, natamycin concentrations of 1-25 ug/ml (Pimaricin, Haorui Pharma-Chem, Edison, NJ, USA) and 1-10 ug/ml (Natacyn) usually inhibit *Aspergillus, Candida, Cephalosporium, Curvularia, Fusarium, Penicillium, Microsporum, Epidermophyton, Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum,* and *Sporothrix schenckii.*[7] It also has some activity *in vitro* and *in vivo* against *Trichomonas vaginalis.*

Two brands of natamycin ophthalmic suspension are available in the Philippines: Natacyn 5% (Alcon Laboratories, Fort Worth, TX, USA) priced at PhP22,000/15 ml and Elmycin 5% (Elder Group, Mumbai, India) costing PhP150/3ml. This study compared the two brands *in vitro* to determine if the cheaper brand has the same drug concentration and effficacy.

METHODOLOGY

Both brands of natamycin 5% suspension were subjected to antifungal susceptibility testing by broth microdilution. The MIC and MFC were determined as follows.

Ten-milliliter solutions were prepared in Pyrex test tubes by adding 1% chemically pure H2SO4 and 1% chemically pure BaCl2 in increasing amounts. The test tubes were covered with rubber stopper, sealed with melted paraffin, and labeled 1 to 10.

Five-day-old cultures of *Aspergillus flavus, Fusarium solani,* and *Candida parasilopsis* were used as test organisms. These were prepared in Sabouraud's broth and agar slant. A standard inoculum containing about 300,000,000 cells was prepared based on the McFarland tubes (tube number 1). A 0.1cc of the standard inoculum was inoculated in the experimental and control tubes (about 3,000,000 cells/10 ml).

All the tubes were incubated at 25-degree-Celsius room temperature for 6 weeks and shaken for 10 minutes daily to make sure that the inoculum gets uniform contact with the antifungal agent. Three test tubes were prepared for each concentration level.

To confirm the presence of growth in each test tube, all Sabouraud's broth tubes were sampled weekly and streaked in a Sabouraud's agar, which was prepared by mixing 40g/L dextrose, 10g/L neopeptone, and 15g/L agar-agar. These slants were incubated at 25° Celsius for 6 weeks. Once growth was confirmed, a culture mount was done from all the slants that grew organisms to check if the samples collected were similar to the test organisms.

The drug-free control and the drug-inoculum tubes were observed and compared for possible signs of growth daily for 6 weeks. The MIC (the highest concentration with positive fungal growth after six weeks incubation) and the MFC (the lowest concentration where there is no growth after 6 weeks incubation) were determined for each drug.

A confirmatory test for MFC concentration was done by subjecting the tubes with negative growth to centrifugation, washing the sediments three times with sterile NSS, and culturing the washed sediments in Saboraud's dextrose agar. A negative growth after 6 weeks incubation period confirmed the death of the organism.

Student *t*-test for paired samples was done.

RESULTS

Results of the tube dilution against *Aspergillus flavus* showed no growth for concentrations of 150-40000 g/ml for both Natacyn and Elmycin at the end of the 6 weeks

observation period. Both had the same MFC (150 µg/ml) and MIC (75 µg/ml) and showed positive growth (all 3 test tubes for Elmycin and 1 of 3 for Natacyn) on day 7. On day 13, all three test tubes of Natacyn were positive for growth. All concentrations below the MIC showed positive growth in the first week of observation period for both brands. *T*-tests for paired samples showed no significant difference between the MIC and MFC at 150mg/ml of both brands at α=0.05.

For *Fusarium solani,* both the MFC (10µg/ml) and MIC (5µg/ml) were similar. All test tubes with this concentration showed signs of growth on day 1 of the observation period.

For Candida parasilopsis, both brands remained negative until the end of six weeks at concentrations 300 to 4000µg/ml. Both had the same MFC (300µg/m) and MIC (150µg/ml) and showed positive growth concentration on day 12.

DISCUSSION

No difference in the MIC and MFC were noted between Natacyn and Elmycin in all the test fungi (*Aspergillus flavus, Fusarium solani,* and *Candida parasilopsis).* The MIC and MFC were lowest with *Fusarium solani* for both brands. Results for all the test tube replicates were consistent for both brands except in *Aspergillus flavus,* which showed a slight difference betwen the means of each brand. *T*-test paired samples of weekly results, however, showed that the difference was not significant. The results with *Fusarium solani* and *Candida parasilopsis* were exactly the same.

Natacyn and Elmycin have the lowest *in vitro* MIC (5µg/ml) in *Fusarium solani* compared with amphotericin B (20mg/µl. Clinically, the cure rate for fungal keratitis caused by *Fusarium solani* is higher with topical natamycin than with amphotericin B. Nakamura et al. reported a cure rate of 16 out of 18 culture-proven *Fusarium* corneal ulcers for natamycin 5% suspension applied hourly compared with only 7 out of 20 cases for amphotericin B.[8] These results were reflected in a larger series where natamycin 5% suspension had a cure rate of 29 out of 35 *Fusarium* keratitis cases.[9]

Amphotericin B is the drug of choice for treatment of infections resulting from *Coccidioides immitis, Histoplasma capsulatum, Cryptococcus neoformans, Blastomyces dermatitidis, Candida* species, and other less common fungi. The extent to which it can damage the cell wall is dose-related. However, a more rapid death of the yeasts cannot be achieved clinically by increasing the drug dosage because the same cytoplasmic membrane damage also affects human cells, causing unpleasant and potentially dangerous side effects which are almost inevitable even at theraputic levels. Systemic side effects include renal damage, anemia, nausea, vomiting, GI cramps, and diarrhea. Topical application of 1% amphotericin B in a Deoxycholate vehicle causes progressively worsening corneal epithelial defects, stromal opacities, and severe iridocyclytis.[10]

The MIC of Elmycin for *Fusarium solani* was comparable with those of Primaricin and Natacyn. However, its MIC for *Aspergillus flavus* (75µg/ml) and *Candida parasilopsis* (150µg/ml) was higher compared with MIC or Primaricin reported by Mauger (1-25µg/ml).[7] This could be explained by the differences in the antibiotic responses of different species and strains of fungi. The lower MFC of Elmycin and Natacyn for molds (*Fusarium* and *Aspergillus*) vis-à-vis yeasts (*Candida*) may be accounted for by the difference in the cell-wall thickness of the organisms. Cell walls of yeasts are generally thicker (300 nm) than that of molds (200 nm), making it difficult for the drug to bind to ergosterol in susceptible cellular membranes, altering membrane permeability and inducing electrolyte imbalance with resultant cell death.[11]

This is an *in vitro* experiment to determine the minimum drug concentration at which two brands of natamycin can inhibit or kill the fungi. Its results may not correspond with *in vivo* clinical scenario because of host factors, corneal penetration of the antifungal, and difficulty in standardizing antifungal sensitivities. Several studies have shown that natamycin was not effective for deeper stromal involvement and that drug absorption depends on the state of damage to the epithelium.

However, based on the *in vitro* study, Elmycin may be used as a cheaper alternative agent against fungal keratitis, particularly if caused by *Fusarium solani.*

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