A COMPARATIVE STUDY OF MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES OF AMPHOTEREcin – B NAD NATAMYCIN FOR FUSARIUM ISOLATES ON SABOURAUD’S DEXTROSE BROTH (SDB) AND RPMI - 1640 BY BROTH MICRODILUTION METHOD

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ABSTRACT

A total of 180 samples from infected cornea were obtained between the periods of September 2008 to August 2009, from a tertiary care eye hospital. A total of 103 fungal isolates were obtained from the processing of the above samples and 60 isolates were confirmed as Fusarium spp. based on microscopy and cultural characteristics. Amphotericin B and Natamycin were evaluated for their minimum inhibitory concentration (MIC) for Fusarium spp., by broth microdilution method using RPMI-1640 and SDB media. The strains differed in their antifungal susceptibility against the drugs. And there was a slight variation with regard to the influence of effect of media.

Key Words: Keratitis, Fusarium spp., Minimum Inhibitory Concentration, Amphotericin B, Natamycin, RPMI – 1640 and SDB.
INTRODUCTION

Corneal infections due to fungi are very common and represent 30% to 40% of all culture positive infectious keratitis cases in south India. (Bharathi et al., 2003) At least 70 genera of fungi have been associated with fungal keratitis. Of these, *Fusarium* and *Aspergillus* species are responsible for as many as 70% of these cases. (Aggarwal et al., 1994) *Fusarium* is one among the common fungal pathogens causing fungal keratitis (Gopinathan et al., 2002). It is a filamentous fungus widely distributed in soil, plants and different organic substrates. *Fusarium* species is important as plant pathogens, but during recent years they are increasingly associated with humans and now represent the second most frequent mold that causes invasive fungal infections in immunosuppressed patients and is associated with high morbidity and mortality (Consigny et al., 2003). Its incidence appears to be increasing over the last 15 years and the mortality rates due to fusariosis is as high as 80% due to the resistance of the fungus to present antifungal agents (Guarro et al., 1995). The different groups of antifungal agents used for treatment of filamentary keratitis include polyenes such as natamycin and amphotericin B; triazoles like econazole, voriconazole; clotrimazole, imidazole, fluconazole and echinocandins are the recommended therapy (Thomas, 2003). As newer antifungal agents become available, efforts to standardize susceptibility testing methods for fungi have been undertaken (Papithou et al., 2002).

Even though susceptibility testing is used more frequently in bacterial than in fungal infections, it is gaining credibility and interest these days in literature. In the ophthalmic literature very less has been done to investigate the susceptibility of fungi against antifungal preparations (Prajna et al., 2007). Therefore studying the susceptibility profiles of *Fusarium* spp. could be helpful in choosing the best antifungal therapy for the treatment of the infection. The present study was undertaken with the following objectives: to isolate and characterize *Fusarium* species from corneal specimens and to investigate the *in vitro* susceptibility of *Fusarium* spp. that were isolated from fungal keratitis patients against Amphotericin B and Natamycin.
preparations on Sabouraud's Dextrose Broth (SDB) and RPMI-1640 media by broth microdilution method.

MATERIALS AND METHODS

The specimen’s viz., corneal scrapings and corneal swabs were collected from suspected cases of keratitis after a thorough examination by an ophthalmologist. The specimens were collected by using a sterile Kimura's spatula aseptically under slit lamp illumination after administering a local anesthetic such as lignocaine or xylocyne by a trained laboratory technician. The scraped out material was inoculated directly onto solid media such as blood agar and Sabouraud's dextrose agar (SDA) in a row of C-shaped streaks. The scrapings were also placed onto clean, scratch free, labeled slides in a thin uniform manner for 10% KOH wet mount, Gram staining, Giemsa staining and Kinyoun's staining procedures. The techniques were carried out carefully exerting extreme care and caution. The isolates on SDA were further cultured on Potato Dextrose Agar. The isolates were also subjected to microscopic identification using lacto phenol cotton blue staining. For antifungal susceptibility testing, broth microdilution method was followed according to the recommendations of the National Committee for Clinical Standards (NCCLS, 1998) M38-P document which is a standard reference method for testing the antifungal susceptibility of filamentous fungi that cause invasive fungal infections including Fusarium species. Inocula were prepared according to the M38-A document. Following incubation at 35 °C for 48 hours, Minimum Inhibitory Concentration (MIC) was determined according to the NCCLS M38-P document. The drugs available in the form of eye drops in commercial medical shops were used: Amphotericin B (0.15 % Aurolabs, Madurai, India) and natamycin (Natamet, 5% suspension, Sun Pharmaceutical Ind. Ltd., India) were used for the study.
RESULTS AND DISCUSSION

A total of 180 samples from infected cornea were obtained between the period of September 2008 and August 2009. On Gram staining 77 samples were found positive for the presence of Gram positive cocci, rods and Gram negative rods. On direct microscopic examination by KOH wet mount, fungal mycelia were observed in 103 samples. Out of 103 isolates analysed 60, 32 and 11 isolates were identified as *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp., respectively (Table 1). The *Fusarium* isolates obtained were designated as FS1-FS60.

Table 1: Fungal isolates from keratitis

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Genera</th>
<th>No. of Isolates</th>
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<tr>
<td>1</td>
<td><em>Fusarium</em> sp.</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td><em>Aspergillus</em> sp.</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td><em>Curvularia</em> sp.</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>103</td>
</tr>
</tbody>
</table>

*Fusarium* spp., was the commonest fungal isolates with 58.2% occurrence followed by *Aspergillus* spp., with 31%. Several other studies in India reported related results (Dunlop *et al.*, 1994 and Sharma and Athmanathan, 2002). Studies from tropical areas such as Thailand, Hong Kong, Singapore, Paraguay, Florida and Tanzania reported that *Fusarium* spp., as the most predominant fungal pathogen (Mino *et al.*, 1991; Panda *et al.*, 1997; Mselle, 1999; Houang *et al.*, 2001; Tasanee *et al.*, 2008; Boonpasart *et al.*, 2002), whereas *Aspergillus* spp., were the most predominant fungal pathogen in the temperate regions of north, west and east India, Nepal and Bangladesh (Chander and Sharma, 1994; Despande and Koppiar, 1999; Samar *et al.*, 2005; Upadhyay *et al.*, 1991; Yeh *et al.*, 2006; Sherwal and Verma, 2008). This variation may be explained by difference in the climatic condition and the natural environment.
The MIC of Amphotericin B for the 60 isolates varied between 2 μg/ml to 32 μg/ml. A total of 19 (32%) and 22 (36%) isolates showed an MIC value of 8 μg/ml on both RPMI-1640 and SDB. MIC\textsubscript{30} and MIC\textsubscript{90} values of Amphotericin B for the isolates were 4μg/ml & 16 μg/ml and 8 μg/ml & 32 μg/ml in RPMI-1640 and SDB respectively (Fig.1). According to the CLSI guidelines, fungal isolates inhibited between MIC ranges of 0.5 and 4 μg/ml are considered to be susceptible. Amphotericin B has been the mainstay of therapy since the 1950s and was the only antifungal drug available. Recently, alternatives that are less toxic and more easily administered than amphotericin B have been developed. At the same time, there are increasing numbers of reports indicating the emergence of resistance to the new agents (Barchiesi \textit{et al}., 1994; Rex \textit{et al}., 1995; White and Goetz, 1994). Amphotericin B alone or in combination with flucytosine or rifampin is the mostly commonly used antifungal drug for the treatment of systemic fusariosis (Sampathkumar and Paya, 2001). Rotowa \textit{et al} (1990) tested 18 strains and reported MIC\textsubscript{90} values of 4 mg/L and in our study MIC\textsubscript{30} and MIC\textsubscript{90} values of Amphotericin B for the isolates were 4 μg/ml & 16 μg/ml and 8 μg/ml & 32 μg/ml in RPMI-1640 and SDB respectively.

Figure 1  MIC values of Amphotericin B for \textit{Fusarium} sp., on RPMI-1640 and SDB
The MIC of natamycin for the 60 isolates of *Fusarium* spp. was found to range between 8 and 64 μg/ml on both RPMI-1640 and SDB. Of 60 isolates, 44 % and 50% of the isolates had MIC of 16 μg/ml on RPMI-1640 and SDB respectively for Natamycin. And 5% of the isolates had MIC of 64 μg/ml in both the media against Natamycin. MIC 30 and MIC 90 values of Natamycin for the isolates were 16μg/ml & 32μg/ml in both RPMI-1640 and SDB (Fig.2).

Figure 2 MIC values of Natamycin for *Fusarium* sp., on RPMI-1640 and SDB

In a study conducted by Lalitha *et al.* (2008), Natamycin and Amphotericin B had good activity against *Fusarium* spp., Further voriconazole, amphotericin B and posaconazole had the lowest MIC against *Fusarium* spp., and none of the *Fusarium* spp., were inhibited by itraconazole or caspofungin (Lalitha *et al.*, 2007). In a recent study from China on the pattern of ocular fungal isolates it was found that *Fusarium* spp., was the predominate pathogen and the 93% of isolates were sensitive to natamycin (Xuguang *et al.*, 2007). To date, susceptibility break points have not been established for natamycin but the MICs obtained are likely within the achievable levels obtained in the eye during standard therapy. Lalitha *et al.* (2008) examined the
susceptibility of 41 isolates of *Fusarium* sp. from keratitis to natamycin and the MIC 90 value was 4µg / ml in contrast to our finding of 32µg/ml in both RPMI-1640 and SDB. 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 natamycin (Xuguang *et al*., 2007).

Topical natamycin is used for the treatment of keratitis due to *Fusarium* spp., (Rahman *et al*., 1998). In Singapore, topical natamycin 5% and topical amphotericin B (1 mg/ml) are the first-line treatment for suspected fungal keratitis because *Fusarium* spp., and *Aspergillus* spp., are the most commonly isolated organisms locally (Wong *et al*., 1997a). Topical natamycin 5% has been shown in one study to be superior to topical itraconazole 1% in the management of fungal keratitis due to filamentous species including *Fusarium* spp., (Kalavathy *et al*., 2005).

Overall, the determination of MICs of the test antifungal drugs against a part of test fusaria (n = 60) was noticed to be useful in understanding/validating the efficacy of increased concentrations of the drugs in inhibiting the germination of fungal spores. Remarkably, isolates of fusaria showing resistance to most of the antifungals tested in the present study were noted to be common. Since, one of the root causes of such an antifungal resistance is empirical therapy; the study underscores routine testing/interpretation of the isolated *Fusarium* strains for their antifungal susceptibility through standard methods. This would bring-in appropriate strategies of therapy and would prevent further emergence and dissemination of drug resistant fusaria in this region.