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Original Article

ANTIFUNGAL SUSCEPTIBILITY TESTING TO DETERMINE MIC OF AMPHOTERICINE B, FLUCONAZOLE AND KETOCONAZOLE AGAINST OCULAR FUNGAL INFECTION

Mitesh H. Patel[1], Avani M. Patel[2], Sachin M. Patel[3], Govind L Ninama[4], Kamlesh R. Patel[5], Bharti C. Lavingia[5]

[1]Associate professor, Department of Microbiology, Medical College & SSG Hospital, Vadodara [2]Tutor, Department of General Pathology & Microbiology, Govt. Dental College, Ahmedabad [3]Tutor, Department of Microbiology, B J Medical College, Ahmedabad [4]Assistant professor, Department of Microbiology, Medical College & SSG Hospital, Vadodara [5]Consultant Microbiologist, Sarthak Laboratory & Cornea foundation, Ahmedabad

Correspondence:
Dr. Mitesh H. Patel
21, Jankinandan Society,
Nava Vadaj, Ahmedabad - 13.
Email: drmitesh_76@yahoo.co.in Mobile: +91-9825171815

ABSTRACT

Purpose: To standardize In-vitro antifungal susceptibility testing by agar dilution method to find out minimum inhibitory concentration (MIC) of Amphotericine B, fluconazole and ketoconazole against ocular fungal isolates.
Methods: A total of 90 isolates (25 yeast & 65 filamentous fungi) were included. The drugs were added in serial double dilution in medium containing yeast nitrogen base. The MIC was determined by inhibition of visible growth on lowest concentration of drug containing media as comparing with visible growth on drug free media.
Results: Out of 25 yeast isolate, all are sensitive to Amphotericine B, 8 % & 12 % are resistant to Fluconazole & ketoconazole respectively. Out of 65 Filamentous fungi, 3 %, 20 % & 28 % are resistant to Amphotericin B, Fluconazole & Ketoconazole respectively.
Conclusion: The methodology used in study is user's friendly, reliable & cost effective.

Key words: Yeast, Mold, MIC

INTRODUCTION

Fungi rarely infect healthy & intact ocular tissue. Fungi, nowadays, are emerging as an opportunistic pathogen of keratitis & endopthalmitis in developing countries. Fungi are major etiological agents of corneal ulcer (44%) in India.[1] Mycotic keratitis occur at any age, but highest incidence coincides with period of maximal activity. Men are involved twice as often as women and agricultural workers & outdoor labourers constitute the biggest occupational group. Minor injury to the eye is an important predisposing factor.[2] *Fusarium* species (47.1 %) & *Aspergillus* species (16.1 %) are the most common etiological agents of corneal ulceration in India & Natamycine & Amphotericine B remains the drug of choice for superficial fungal keratitis.[3]

Fungal keratitis contributes to 6 – 56 % of keratitis.[4] Fungal endopthalmitis contributes to 4 -11 % of endopthalmitis.[5] Epidemiological trend is different as per country to country & region to region. Increasing number of non responding ocular fungal infection need for antifungal susceptibility testing. The need increase beyond testing *Candida* species because resistance to

antifungal drugs have been demonstrated against such diverse fungi as *Cryptococcus neoformans, Aspergillus* spp., *Histoplasma* & *Trichosporon* species. So need for susceptibility testing is very important for fungi as it is for bacteria. At present in all over world, antifungal susceptibility testing is less standardized & established than antibacterial testing.[6]

The methods include Clinical laboratory Standards Institute, CLSI, broth based methodology (M 27-A), CLSI methodology for moulds, E-test agar based testing methods, flow cytometry & use of viability dyes. The above methods are time consuming & labor intensive, hence a more economical method such as Agar dilution have been used. There are limited number of such study are done in India.

MATERIALS & METHODS

The study was carried out with the ocular fugal isolates obtained between November 2004 to April 2010 in tertiary care hospital & research institute for Cornea affiliated with dedicated laboratory for Microbiology in western India.

Ocular isolates of fungi included in the study

25 yeast & 65 filamentous fungi were included in the study. It is shown in Table 1. The fungi were isolated by the standard procedures from corneal scrapings of keratitis & endopthalmitis patients. The yeasts were identified by the germ tube test & carbohydrate fermentation & assimilation test. The filamentous fungi were identified based on colony morphology & lacto phenol cotton blue mount preparation of the slide cultures.

Table 1: Ocular yeast isolates

Isolate	Corneal scrapping – 17	DCR – Donor corneal rim – 3	Conj. Swab - 3	Vitreous fluid – 2	Total - 25
C. albicans	7	2	1	1	11
C. tropicalis	2	-	-	1	3
C. parapsilosis	-	-	-	-	-
C. krusei	3	-	1	-	4
C. guilliermondii	-	-	-	-	-
C. lipolytica	3	-	1	-	4
Trichosporon beigellei	1	1	-	-	2
Rhodotorula rubra	1	-	-	-	1

Table 2: Ocular isolates of filamentous fungi

Isolate	Corneal scrapping – 31	Corneal button - 15	DCR – 12	Aqueous humor - 1	Vitreous aspirate – 6	Total – 65
A. flavus	10	3	2	-	1	16
A. fumigatus	8	4	2	-	2	16
A. terreus	2	2	1	-	1	6
A.niger	5	1	4	1	2	13
Fusarium species	4	2	1	-	-	7
Penicillium species	2	-	1	-	-	3
Paecilomyces species	-	2	1	-	-	3
Dematiaceous fungi	-	1	-	-	-	1

Stock solutions of all three antifungal drugs were prepared by drug in suitable solvent. Yeast nitrogen base was used as a culture media. The medium was prepared in sterile screw capped glass bottles (15 ml). Required concentrations of antifungal drugs were incorporated in medium. Along with that control set of medium without drugs were inoculated.

Fungal isolates grown over sabouraud dextrose agar were suspended in sterile distilled water & make a suspension equivalent to 10^5 cells / ml.

Interpretation of result

Plates were inoculated with standard inoculums & incubated at 25 degree centigrade for 48 hour. Afterward, readings were taken. The MIC was

the lowest concentration of drug preventing growth of macroscopically visible colonies on drug containing plates at the time when there was visible growth on drug free control plates.

RESULTS

Out of 25 yeast isolates, none of the isolates show resistant to Amphotericin B. Only 2 isolates show resistant to fluconazole & 3 were resistant to ketoconazole.

Out of 65 filamentous fungal isolates, 2 isolates were resistant to Amphotericin B, while 13 isolates were resistant to fluconazole & 18 were resistant to ketoconazole.

Table 3: Sensitivity pattern of fungal isolates

	Amphotericin B		Fluconazole		Ketoconazole	
Isolate	Sensitive -	Resistant -	Sensitive -	Resistant –	Sensitive -	Resistant –
Yeast – 25	25(100%)	0(0%)	23(92%)	2 (8%)	22(88%)	3(12%)
C. albicans	11	0	10	1	11	0
C. tropicalis	3	0	3	0	2	1
C. parapsilosis	-	-	-	-	-	-
C. krusei	4	0	3	1	2	2
C. guilliermondii	-	-	-	-	-	-
C. lipolytica	4	0	4	0	4	0
Trichosporon beigellei	2	0	2	0	2	0
Rhodotorula rubra	1	0	1	0	1	0
Filamentous fungi – 65	Sensitive – 63(97%)	Resistant- 2(3%)	Sensitive – 52(80%)	Resistant – 13(20%)	Sensitive- 47(72%)	Resistant - 18(28%)
A. flavus	15	1	13	3	11	5
A. fumigatus	16	0	12	4	13	3
A. terreus	5	1	5	1	3	3
A.niger	13	0	11	2	11	2
Fusarium species	7	0	7	0	3	4
Penicillium species	3	0	2	1	2	1
Paecilomyces species	3	0	2	1	3	0
Dematiaceous fungi	1	0	0	1	1	0

DISCUSSION

The present study was designed to know prevalence of fungal pathogen in ocular infection & their susceptibility to various antifungal drugs. Corneal infection of fungal etiology is very common and may represent 30 – 40 percent of all cases of culture-positive infectious keratitis. Of these, *Aspergillus* and *Fusarium* are responsible for 70 percent of cases.[7]

We found that majority of fungus are susceptible to Amphotericin B, followed by Fluconazole & ketoconazole. The CLSI broth micro-dilution method is time consuming, expensive & technically difficult to perform. On the other hand, agar dilution method has two important advantages over CLSI method. The first is visual reading based on intensity of growth showing the clear end point of inhibition. Second is that susceptibility testing of large number of fungi is easier & economical as four strains can be tested with one plate of agar plates in the agar dilution method whereas each strain needs one set of tubes in the CLSI method.

The emergence of antifungal resistance has made susceptibility testing important and applicability of in-vitro antifungal sensitivity testing directly correlated with clinical outcome.

A. flavus & A. terreus are resistant to Amphotericine B. These finding were correlated with the study conducted by Arikan et al in which resistance to Fluconazole & ketoconazole.[8]

In present study, *A.fumigatus* shows highest resistant to fluconazole, while A. flavus shows highest resistant to ketoconazole. Among the yeast candida krusei is emerged as highly resistant yeast, which is comparable to other published data.[9] However, these results are only meaningful when compared to the ocular tissue concentrations of the drugs obtained after oral, topical & parenteral administration.

Aspergillus accounts nearly for 50 % of the reported cases of mycotic keratitis & is the most frequent fungus in many parts of the India.[10] *Aspergillus spp*. were the most common isolates (39.5%) in mycotic keratitis followed by *Fusarium* (10.7%), *Alternaria* (10.2%), *Curvularia* (7.4%) and *Penicillium* (7%). Trauma was the most common predisposing factor (55.3%) followed by associated systemic illness (11.2%), previous ocular surgery (9.8%) and others.[11] Of 360 cases of clinically suspected suppurative keratitis, amongst positive isolates, 79% were bacterial & 16% fungal (predominantly *candida*) & *Acanthamoeba*, according to an Australian study.[12]

Antifungal susceptibility should reliably predict the *in vivo* response to therapy in human infections. However, drug pharmacokinetics & drug interactions, factors related to the host immune response and the status of current underlying disease, proper patient management system & factors relates to virulence of infecting organism & its interaction with both the host & therapeutic agents appear to have more value than the MIC as predictors of clinical outcome.

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