Original Research Article

In vitro antifungal susceptibility testing of ocular filamentous fungal pathogens isolated from keratitis cases

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Abstract

Background: A variety of fungal species are known to cause keratitis. Filamentous fungi are often predominant cause of keratitis. As fungi belonging to different group usually differ in their pattern of susceptibility to antifungal agents commonly prescribed for mycotic keratitis, *in vitro* susceptibility testing usually guide ophthalmologists for selection of most appropriate agent from available antifungal armamentarium. The present study was conducted in a tertiary care teaching hospital with an aim to study antifungal susceptibility profile of ocular filamentous fungi. **Material and Methods:** Filamentous fungi were identified on the basis of macroscopic features of colony and microscopic characteristics. Antifungal susceptibility was performed exactly as per the methodology outlined in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document for antifungal susceptibility testing of filamentous fungi. **Results:** Among fungal isolates, 72 (77.4%) isolates were filamentous fungi whereas 21(22.6%) were yeasts. *Fusarium* spp. (43.1%) and *Aspergillus* spp. (29.2%) were common filamentous fungi isolated from keratitis cases. All filamentous fungi isolated from keratitis cases were susceptible to amphotericin B. *Aspergillus* spp. and *Bipolaris* spp. were resistant to natamycin. **Conclusion:** As inter and/or intra species variation in susceptibility to commonly used antifungal drugs do occur in ocular filamentous fungal pathogens, the present study highlights the importance of antifungal susceptibility testing of each and every isolate from mycotic keratitis cases.

Keywords: Mycotic keratitis, *Fusarium* spp., Natamycin, Amphotericin B, Fluconazole.

Introduction

Mycotic keratitis is an infection of corneal stroma caused by a diverse species of fungi[1]. It is frequent cause of visual disability and the 2nd most cause of blindness after cataract[2]. Risk factors like ocular trauma, ophthalmic surgeries, use of contaminated ophthalmic solutions, use of contact lens and diabetes mellitus are contemplated for mycotic keratitis[3].

Usually causes of mycotic keratitis in developed and developing countries are different. In developed countries the use of contact lens is more associated with mycotic keratitis whereas in India and most of developing countries ocular trauma with vegetative matter contaminated with fungi is identified as a major cause[4].

A variety of fungal species are known to cause keratitis[5]. These fungi are mostly saprophytic in nature and prevalent in air, soil, vegetative material and water[6]. Etiological agents of fungal keratitis vary among different regions[5]. These include hyaline or dematiaceous filamentous fungi and *Candida*.In most though not all studies on mycotic keratitis, predominance of filamentous fungi was noted.

As fungi belonging to different group usually differ in their pattern of susceptibility to antifungal agents commonly prescribed for mycotic keratitis, *in vitro* susceptibility testing usually guide ophthalmologists for selection of most appropriate agent from available antifungal armamentarium[7].

Compared to *Candida* spp., antifungal susceptibility testing of filamentous fungi is seldom performed in most of clinical microbiology services. The present study was conducted in a tertiary care teaching hospital with an aim to study antifungal susceptibility profile of ocular filamentous fungi.

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Material and methods

The present cross-sectional, descriptive study was conducted in the Department of Microbiology, Government Medical College, Aurangabad, Maharashtra. Filamentous fungi isolated from cases of keratitis were included in the study. For demonstration of fungal elements and isolation of fungi from keratitis cases, corneal scrapings collected by ophthalmologist were used. 10% potassium hydroxide (KOH) mount was used for demonstration of fungal elements.

For culture, corneal scrapings were inoculated blood agar, chocolate agar and two sets of Sabouraud dextrose agar (SDA) with chloramphenicol.[4]C-shaped streak was made on culture plates to ensure that growth was from the specimen and not of laboratory contaminants. One set of SDA was incubated at 25°C while other at 37°C. Blood agar and chocolate agar plates were examined daily for seven days and discarded if no growth was observed. SDA plates were examined daily for twenty one days. Standard bacteriological and mycological protocols were used for identification of microbial growth.

Filamentous fungi were identified on the basis of macroscopic features of colony (color and texture) and microscopic characteristics by observing lacto-phenol cotton blue(LPCB) mount and slide culture[8].

Ocular filamentous fungi were tested against commonly used antifungal drugs like Fluconazole (range 0.125 to 64 µg/ml), Amphotericin B (range 0.03 to16 µg/ml) and Natamycin (range 0.25 to128 µg/ml). Antifungal drugs in powder form were procured from commercial sources.

Antifungal susceptibility was performed exactly as per the methodology outlined in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document for antifungal susceptibility testing of filamentous fungi[9].

A complete synthetic medium RPMI-1640 medium (with glutamine and phenol red, without bicarbonate) supplemented with 0.2% glucose and buffered to a pH of 7.0 with 0.165 mol/L MOPS (3-[N-morpholino] propanesulfonic acid) was used.

Before antifungal susceptibility testing, filamentous fungi were grown on potato dextrose agar at 37°C for approximately 7 days. *Aspergillusniger* ATCC 16404 was included as a quality control strain.

End point determination was done as per the CLSI M38-A2 document. The minimum inhibition concentration (MIC) (the lowest concentration of the drug that inhibits growth of the organism) was determined. The amount of growth in the tubes containing the antifungal agent was compared to that of the growth in the growth-control tubes used in each set of tests.The growth was scored by visual inspection as follows: 4-no reduction in growth; 3-slight reduction in growth or approximately 75% of the growth control; 2-prominent reduction in growth or approximately 50% of the growth control; 1-slight growth or approximately 25% of the growth control; 0-optically clear or absence of growth[9].For amphotericin B, the MIC was read as the lowest concentration of drug that resulted in 100% reduction in turbidity as compared to drug-free control tubes or numerical score 0. For fluconazole and natamycin the MIC was read as the lowest concentration of drug that exhibited 80% reduction in turbidity or numerical score 2[9].

Results

During the study period (3 years), a total of 267cornealscrapings were received in the Department of Microbiology from suspected cases of keratitis. Out of these, a total of 148 (55.4%)showed microbial growth. These included 93 (62.8%) fungal and 55 (37.2%) bacterial isolates (Figure 1). In the present study, fungi weresignificant cause of keratitis (Fisher exact test $P<0.05$).

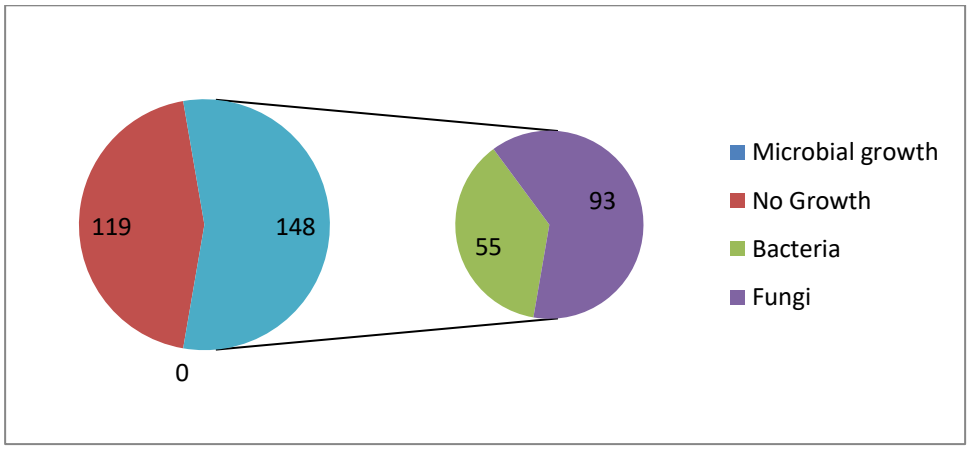

Fig 1: Microbial growth from corneal scrapping.

Among fungal isolates, 72 (77.4%) isolates were filamentous fungi whereas 21(22.6%) were yeasts. *Candida* was the only yeast type isolated from cases of keratitis. Filamentous fungi isolated from keratitis are shown in table 1.*Fusarium* spp. (43.1%) and*Aspergillus* spp. (29.2%) were common filamentous fungi isolated from keratitis cases. Among *Aspergillus* spp., *A. niger* was the most common.

Table 1: Filamentous fungi isolated from keratitis.

Filamentous fungi	No. (%)
Alternaria spp.	04 (5.6)
Aspergillusspp.	21 (29.2)
A. flavus	04
A. fumigatus	03
A. niger	10
A. nidulans	04
Bipolarisspp.	05(6.9)
Fusariumspp.	31 (43.1)
Mucor	05 (6.9)
Pseudallescheriaboydii	04 (5.6)
Penicillium spp.	02 (2.8)
Total	72

Antifungal susceptibility profile of filamentous fungi isolated from keratitis cases is shown in table 2.*Aspergillus*spp. Mucor, *Bipolaris* spp. and *Pseudallescheriaboydii* were resistant to fluconazole. As *Pseudallescheriaboydii* is intrinsically resistant to amphotericin B, it was not tested by amphotericin B susceptibility. All filamentous fungi isolated from keratitis cases were susceptible to amphotericin B. Aspergillus spp. and Bipolaris spp. were resistant to natamycin.

Table 2: Antifungal susceptibility profile of filamentous fungi isolated from keratitis cases.

Filamentous fungi	Antifungal agent (range in µg/ml)	Geometric mean	MIC 50/ 90
Alternaria spp. (N=4)	Fluconazole (0.125 to 64)	1.9	NA
	Amphotericin B (0.03 to16)	0.6	NA
	Natamycin (0.25 to128)	4	NA
Aspergillus spp. (N=21)	Fluconazole (0.125 to 64)	64	64/64
	Amphotericin B (0.03 to16)	0.5	0.5/1
	Natamycin (0.25 to128)	128	128
Bipolaris spp. (N=5)	Fluconazole (0.125 to 64)	32	NA
	Amphotericin B (0.03 to16)	1	NA

	Natamycin (0.25 to128)	128	NA
Fusariumspp. (N=31)	Fluconazole (0.125 to 64)	2	2/2
	Amphotericin B (0.03 to16)	0.24	0.24/0.48
	Natamycin (0.25 to128)	4	4/8
Mucor (N=5)	Fluconazole (0.125 to 64)	64	64/64
	Amphotericin B (0.03 to16)	0.06	0.06/0.12
	Natamycin (0.25 to128)	8	8/8
Pseudallescheriaboydii (N=4)	Fluconazole (0.125 to 64)	32	NA
	Amphotericin B (0.03 to16)	Not tested	
	Natamycin (0.25 to128)	8	NA
Penicillium spp. (N=2)	Fluconazole (0.125 to 64)	1	NA
	Amphotericin B (0.03 to16)	0.12	NA
	Natamycin (0.25 to128)	0.5	NA

Discussion

Last few decades have witnessed a significant upswing in incidence of mycotic keratitis cases. As per recent data, more thanone million people are affected by mycotic keratitis annually and about 8 to 11% of these patients lose the eye.[3] Diagnosis of mycotic keratitis is often more difficult and has a worse outcome compared to other types of microbial keratitis[10].Although Keratitis is most prevalent in tropical and subtropical countries, an increasing trend is also observed in countries with moderate climates[11]. In India, a favourable tropical climatic condition along with primary agrarian population is important predisposing factor for mycotic keratitis[12]. Various research studies have documented isolation of mycotic agents in 20-60% of culture proven cases of keratitis.Similarly in the currentstudy, out of 148 culture proven cases,fungi were isolated from 72 (62.8%) corneal scrappings. In most of cases, mycotic keratitis occur secondary to ocular trauma due to variety of organic material. Mycotic keratitis is common among adults, predominantly males[2-4].Although more than 100 fungal species are implicated in keratitis, 95% of cases are attributed to *Aspergillus* spp., *Fusarium* and *Candida*[3]. In the present study predominance of filamentous fungi (77.4%) over *Candida* spp. (22.6%) was noted. In countries with tropical and subtropical climatic conditions, filamentous fungi are responsible for most ofmycotic keratitis whereas in temperate climates yeasts are more common[13].Similar to many other studies on mycotic keratitis, *Fusarium* spp. (43.1%) and *Aspergillus* spp. (29.2%) were common filamentous fungi isolated from keratitis cases in this study. Studies on mycotic keratitis have reported isolation of *Fusarium* spp. and *Aspergillus*spp, in the range of 37-62% and 24-30% respectively[7].In recent years increased isolation of dematiaceous or phaeoid fungi are reported in many studies. In the current study a total of 9 filamentous fungi belonged to dematiaceous fungi group. These included 4 isolates of *Alternaria* spp. and 5 isolates of *Bipolaris* spp. Dematiceous fungi are saprotrophic in nature and their manifestations are usually seen in healthy individuals[14].As keratitis due to filamentous fungi tend to have a worse prognosis compared to those caused by yeast species, an early and accurate diagnosis along treatment with most appropriate antifungal drug is very essential to prevent devastating ocular consequences. A significant variations is observed in susceptibility patterns of inter and/or intra species of ocular fungal isolates. Therefore antifungal susceptibility testing of each and every isolate is necessary for selection of the most effective antifungal agent. Thorough the collaborative efforts of researchers and the CLSI Subcommittee on Antifungal Susceptibility Testing, antifungal susceptibility testing is standardized and plays an important role in guiding the antifungal therapy[15]. However, its utility still remains underutilized compared to antibacterial susceptibility testing. The CLSI published reference method for susceptibility testing filamentous fungi (M38-A) in 2002[9,15]. This M38-A has created a standard for comparison of clinical data. In the present study, the CLSI reference method was determine antifungal susceptibility of ocular filamentous fungi. The ocular fungal isolates were tested against antifungal drugs like Amphotericin B, fluconazole and natamycin.Amphotericin B is a polyene antifungal agentwith broad spectrum activity[16]. In the present study, all filamentous fungi were susceptible to amphotericin B. Although amphotericin B demonstrated good in *vitro* efficacy against ocular fungal pathogens, it has poor penetration in cornea and requires high dosages to achieve the sufficient concentration[17].Natamycin is the first line drug for keratitis in many developing countries[10]. In the present study, ocular filmentous fungi like *Alternaria* spp., *Fusarium* spp., Mucor, *Pseudallescheriaboydii* and *Penicillium*spp. had MICs of≤8 µg/ml for natamycin. Fungal isolates with isolates with MICs of ≤16 µg/ml for natamycinare considered susceptible because at this concentration, natamycinadequately reach the eye during treatment. *Aspergillus* spp. and *Bipolaris* spp. were resistant to natamycin. This finding is accordance to previous studies were *Aspergillus* spp. demonstrated high MIC for natamycin[2]. Rahman*et al*. (1998) reported that natamycineven at higher concentration is ineffective against *Aspergillus* spp[18].Oral fluconazole combined with topical natamycin has been reported as an effective therapeutic modality for keratitis due to filamentous fungi. In the present study, *Aspergillus* spp., *Bipolaris* spp., Mucor and *Pseudallescheriaboydii* were resistant to fluconazole. In the study of Manikandan*et al*. (2013) all isolates of *Aspergillus* were resistant to fluconazole. Many other researchers have reported the similar finding[2].

Conclusion

Filamentous fungi are important cause of keratitis. Rapid and accurate identification of infecting species is extremely necessary to understand epidemiology of keratitis. As inter and/or intra species variation in susceptibility to commonly used antifungal drugs do occur in ocular filamentous fungal pathogens, the present study highlights the importance of antifungal susceptibility testing of each and every isolate from mycotic keratitis cases. Antifungal susceptibility often aid ophthalmologist in selection of most appropriate drug for treatment of keratitis.

References

1. Thomas PA. Current perspectives on ophthalmic mycoses. Clin Microbiol Rev 2003; 16: 730–797.
2. Manikandan P, Varga J, Kocsube S, Raghavan A, Rajaraman R, Nemeth T*et al*.Epidemiology of Aspergillus keratitis at a tertiary care eye hospital in South India and antifungal susceptibilities of the causative agents. Mycoses 2013; 56:26-33.
3. Brown L, Leck A, Gichangi M, Burton M, Denning D. The global incidence and diagnosis of fungal keratitis. Lancet Infect Dis 2021; 21: e49-e57.
4. Deorukhkar S, katiyar R, Saini S. Epidemiological features and laboratory results of bacterial and fungal keratitis: A five-year study at a rural tertiary-care hospital in western hospital in western Maharashtra, India. Singap Med J. 2012; 53:264-267.
5. Hoffman J, Burton M, Leck A.Mycotic keratitis—A global threat from the filamentous fungi. J. Fungi 2021; 7: 273.
6. Thomas P. Fungal infections of the cornea. Eye 2003; 17:852-862.
7. Saha S, Sengupta J, Banerjee D, Saha S, Khetan A, Mandal S. Systemic Evaluation on Antifungal Susceptibility of Keratitis Associated Fungal Pathogens in Eastern India. J Med MicrobDiagn 2014; 3:1.
8. Koneman EW, Allen SD, Janada WM, Schrckneberger PC, Winn WC. Colour atlas & Textbook of diagnostic

Microbiology. 6th edition. Philadelphiae; Lippincott-raven publisher; 2006.
9. CLSI. Reference method for broth dilution antifungal susceptibility testing of conidial-forming filamentous fungi; approved standard CLSI M38-A. Wayne (PA): Clinical and Laboratory Standards Institute; 2002.
10. Walther G, Zimmermann A, Theuersbacher J, Kaerger K, von Lilienfeld-Toal M, Roth M *et al*. Eye Infections Caused by Filamentous Fungi: Spectrum and Antifungal Susceptibility of the Prevailing Agents in Germany. J. Fungi 2021; 7: 511.
11. Mahmoudi S, Masoomi A, Ahmadikia K, Rezaie S. Fungal keratitis: An overview of clinical and laboratory aspects. Mycoses. 2018;61:916-930.
12. Chowdhary A, Singh K. Spectrum of fungal keratitis in north India. Cornea 2005;24:8-15.
13. Thomas P, Leck A, Myatt M. Characteristic clinical features as an aid to the diagnosis of suppurative keratitis caused by filamentous fungi. Br J Ophthalmol 2005; 19: 210–220.
14. Garg P, Gopinanthan U, Choudhary K. Keratomycosis: clinical and microscopic experience with dematiaceous fungi. Ophthalmology 2000; 107: 574-580.
15. Fothergill A, Rinaldi M, Sutton D. Antifungal susceptibility testing. Infect Dis Clin N Am 2006; 20:699-709.
16. Kanafani Z, Perfect J. Resistance to antifungal agents: Mechanisms and clinical impact. Clin Infect Dis 2008; 46:120-128.
17. Xie L, Zhai H, Zhao J, Sun S, Shi W, Dong X. Antifungal susceptibility for common pathogens of fungal keratitis in Shandong Province. Am J Ophthalmol 2008;146: 260-265.
18. Rahman M, Johnson GJ, Husain R, Howlader S, Minassian D. Randomised trial of 0.2% chlorhexidinegluconate and2.5% natamycin for fungal keratitis in Bangladesh. Br J Ophthalmol 1998; 82: 919-925.

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