Clinico-microbiological profile of ocular mycosis with special reference to filamentous fungi

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Introduction

Mycotic keratitis presents as a suppurative, usually ulcerative, corneal infection. This entity may account for more than 50% of all cases of culture-proven microbial keratitis and of ophthalmic mycoses, especially in tropical and subtropical areas.

Filamentous fungi are the principal causes of mycotic keratitis in most parts of the world. In studies done around the world, either *Fusarium* spp. or *Aspergillus* spp. were the most common isolates. Dematiaceous fungi, such as *Curvularia* spp. and *Bipolaris* spp. are the third most important cause of keratitis in a number of studies, while the coelomycete *L. theobromane* has been reported to cause keratitis in India and the southern United States.

Most of the causes of blinding corneal pathology are preventable. Proper diagnosis of pathological organism in time can help reduce the unnecessary use of antibacterial or antifungal drugs that might lead to resistance. Corneal trauma is the most frequent and major risk factor for fungal keratitis. In fact, the physician should have a high level of suspicion in a patient with a history of corneal trauma, particularly with plant or soil matter. A history of corneal trauma with vegetable matter or organic matter is reported in 55% to 65% of fungal keratitis, as corneal epithelial layer prevents entry of organism inside the cornea. Fungi gain access into the corneal stroma through a defect in the epithelium, then multiply and cause tissue necrosis and an inflammatory reaction. The epithelial defect usually results from trauma (contact lens wear, foreign material, prior corneal surgery). The fungi can now penetrate an intact descemet membrane and gain access into the anterior chamber or the posterior segment. Mycotoxins and proteolytic enzymes augment the tissue damage. Apart from trauma, decreased host immunity is one of the important causes of fungal infection in cases of diabetic patients, steroid intake topically or orally and intake of any immunosuppressant drugs. Topical steroids as the principal risk factor enhance ocular fungal growth. Steroid use as initial therapy was reported in 1 to 30% of patients having microbial keratitis.

In India, as fungi are the leading causative organism, therefore, rather than following the empirical treatment protocol, it is better to go for identification of the pathogen. It is of utmost importance for the Microbiologists that they try to recognize fungal elements early to expedite the treatment procedure. In India many factors contribute towards the higher incidence of fungal keratitis. India being an agricultural country, most of the corneal trauma is agricultural. The Indian population lacks access to proper eye care, either due to ignorance or lack of facilities to handle eye injury. This leads to rampant use of traditional medicine which are again dried plant material in liquid preparation or animal origin like milk, urine etc., all of which might compound the injury.

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In this study, we have tried to find the risk factors behind development of mycotic keratitis and expedite treatment by giving an early conclusive diagnosis. We tried to find out the demography pattern and occupational susceptibility of the patients. The identification of causative organism and it's susceptibility to commonly used antifungal agents were also studied to see the pattern of resistance based on genus.

Aim

To study clinico-microbiological profile of ocular mycosis with special reference to filamentous fungi.

Material and Method

Processing of slides:

1. **KOH wet mount preparation:** Potassium hydroxide mounting fluid

Distilled water	80 ml
Potassium hydroxide	20gm
Glycerol	20ml

2. **Gram's staining**

3. **Lactophenol Cotton Blue mount preparation:**

Lactophenol cotton blue (LCB) stain:

Phenol	20gm
Lactic acid	20ml
Glycerin	40gm
Cotton blue	0.05gm
Distilled water	20ml

Culture Methods

SDA and blood agar media in petri-dish was used for fungal pathogens isolation. SDA agar supports growth of nearly all fungal pathogens including yeast and filamentous fungi. BA helps growth of bacterial pathogens in the bacterial corneal ulcer.

Antibiotic Sensitivity Testing:

- **Disk diffusion testing for filamentous fungi**

  Commercially prepared paper discs for Fluconazole (25µg/disc), Amphotericin-B (100 units/disc), Itraconazole (10µg/disc) and Voriconazole (1µg/disc), Ketoconazole (10µg/disc) from HiMedia Lab were used.

- **Minimum inhibitory concentration method for filamentous fungi**

  Itraconazole, Voriconazole, Amphotericin B, Fluconazole Ketoconazole were dissolved in DMSO. Drug dilution were prepared following the additive two fold drug dilution scheme to the final concentration for Itraconazole, Voriconazole, Ketoconazole and Amphotericin B, 0.038 to 16µg/ml, for Fluconazole 0.25 to 128µg/ml.

Observation and results

The proposed study was carried out in the Departments of Microbiology and Ophthalmology, Sir Sunder Lal Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi.

We took corneal scrapes from 51 patients excluding obvious bacterial or viral corneal ulcer cases over a period of one year from February 2014 to January 2015. These patients have shown large ulcers with elevated margins, covering more than 2/3rd of cornea with satellite lesions (Fig. 1).

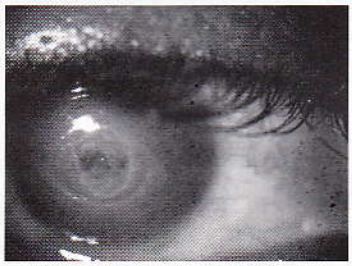

Fig 1. Corneal ulcer

Epidemiological profile of mycotic keratitis:

Demographic profile

Out of 23 positive cases, maximum 48% belonged to age group 41-50. 1 patient belonged to the 11-20 age group and 1 to the >60 age group. 4 patients each belonged to the 31-40 and 51-60 age groups. Rest 2 patients belonged to the 21- 30 age group. Overall male and female numbers were nearly equal (male-12 and female 11).

Residential status

In the culture proven cases 60% patients reside in rural areas. These patients have occupation related to farming and animal handling mainly.

Predisposing risk factors

Right eye (RE) was involved in 57% cases. A history of recent injury was present in 91% cases. In one patient, fungal corneal ulcer had developed after cataract surgery which was performed in a camp. In another case, the patient was suffering from Grave's Ophthalmopathy and the ulcer developed due to exposure. 18 patients (78%) admitted that they have put steroid containing eye drops without consulting any physician. There was no case of contact lens user in our study.

Diagnostic methods and their sensitivity

All corneal scrape material was processed through 20% KOH, gram stain and 1% methylene blue and observed microscopically. Out of 51 cases, in 23 cases fungal hyphae were observed in 10% KOH wet mount preparation after 12 hours (Fig 2).

Fig 2, Fungal hyphae in 10% KOH mount

Gram staining was unable to elucidate fungal hyphae in corneal sample satisfactorily. It gave positive result in 10 (46%) cases only. Methylene blue stained fungal hyphae in 12(52%) cases(Fig 3).

Fig 3, Fungal hyphae in methylene blue stain

The number increased after KOH. But scrape material was not always sufficient in amount to carry out many smear preparations; hence Methylene blue + 10%KOH method was not performed in all cases. Table below is showing sensitivity of all the above methods taking the number of culture positive cases as 100%.

Diagnostic Method	Positive	Negative	%
KOH wet mount	23	0	100
Gram stain	10	13	46
Methylene blue wet mount	12	11	52

Out of these 51 cases, 23(45.1%) were culture positive for filamentous fungi where growth was observed on Sabouraud Dextrose Agar at the point of inoculation. Rest of the cases showed no growth even after 28 days of incubation.

Distribution of fungal isolate

In most of the culture positive cases, growth appeared within 24-48 hours of incubation and confluent growth was observed in 4-5 days. Table below shows the type of isolate and number of each type of isolate found in the study.

Filamentous fungi	No of isolate	%
Aspergillus flavus	7	30
Fusarium spp.	8	35
Curvularia sp	3	14
Bipolaris sp	2	9
Alternaria sp	1	4
Paecilomyces lilacinous	1	4
Unknown	1	4

Colonies of *Aspergillus spp* appeared white at first with small aerial mycelium which turns yellowish green due to pigmentation production. The finding was confirmed by preparation of LCB wet mount where

dichotomously branched hyaline hyphae with characteristic conidiophores were observed. Conidiophores with flask shaped biseriate phialides covering entire vesicle and pointing in all directions belonged to the species *Aspergillus flavus*. Total 7(30%) isolate were *Aspergillus spp.* Further identification through slide culture gave the same result.

In another 8 (34%) cases the isolates were identified as *Fusarium spp.*, they were identified by white cottony growth with purple, orange or brown centre. Dust injury associated with half (50%) of the cases of Fusarium causing mycotic keratitis. In one case, Grave's Ophthalmopathy leading to exposure keratitis by *Fusarium* species was observed.

Antifungal susceptibility testing

Disc Diffusion (DD) Method

In disc diffusion testing, the zone sizes were measured after 48 and 72 hours. *Aspergillus spp.* isolate was highly susceptible to Voriconazole, Itraconazole, Ketoconazole with no resistance at all. Towards Fluconazole 4(57%) isolate were found to be resistant. Amphotericin B was completely unable to prevent the growth of *Aspergillus spp.* isolates except in one case. Voriconazole was the most effective drug with not a single resistant isolate. Fluconazole, Itraconazole and Ketoconazole were ineffective against *Fusarium spp.*

Curvularia species was uniformly sensitive against Voriconazole and Ketoconazole and Itraconazole in DD assay. The Bipolaris *spp.* isolated were sensitive to all the tested antifungals in disk diffusion assay.

Minimum inhibitory concentration method

- Among the *Aspergillus flavus* isolated *Itraconazole* have better action in inhibiting growth with MIC ranging from 0.5-1 in 85.7% isolates. All other antifungals had high MIC values.
- *Bipolaris spp* isolate were resistant to *Amphotericin B, Voriconazole* and *Fluconazole*. Only *Itraconazole* and Ketoconazole were able to inhibit the growth within susceptibility range of MIC <1µg/ml.
- With *Curvularia spp.* isolate no growth was obtained in any of the isolates even after repeating the test twice with modification.
- *Paecilomyces lilacinous* identified in the study was found to be susceptible to *Itraconazole* only with MIC 1µg/ml. All other antifungals were ineffective against this isolate.
- *Itraconazole*, Ketoconazole were sensitive against the *Alternaria spp* isolate with MIC values 1 & 1µg/ml. *Voriconazole* and fluconazole had shown very high MIC of 16 and 32µg/ml.
- Amphotericin B, *Voriconazole* were ineffective in inhibiting growth of *Fusarium spp* with MIC>16 and >16µg/ml respectively in 7 of the cases. *Itraconazole* had MIC ≤1µg/ml against isolates and in one 25% cases ≤8µg/ml. Ketoconazole had MIC of≥8µg/ml against 5 isolates.

Discussion

Corneal infection of fungal etiology *(keratomycoses)* is very common and represents 30-40% of all cases of culture-positive infectious keratitis (Dunlop *et al.*, 1994, Hagan *et al.*,1995, Thomas *et al.*, 2003).[1,2,3] Moreover, fungi have replaced bacteria as the predominant cause of infectious keratitis in developing countries.

Thomas *et al*(2003) had shown that males were affected more commonly with 2.5:1 ratio in comparison to females. In Sharmeen *et al* (2010), 64.63% males were affected. In our study, the male to female ratio is 1.1: 1 with 52% males affected in comparison to 48% of females.

In our study 48% of the patients belong to the 41 to 50 age group. But taking active age group 21-30 and 31-40 into account, we can say roughly 73% of the population belongs to 21 to50 age group. Farming being the primary occupation, this 73% coincides with the productive age of the population of Uttar Pradesh. M Jayhar *et al,*[4] had found that maximum 66.85% of the affected population belong to the 21-50 years age group.

The rural urban population divide is more or less showing the demography of the patient population. Rural population is dependent on farming and animal handling, hence prone to injury to the eye with vegetative matter. In our study, 60% of the affected patients belong to rural areas. Out of this 45% are farmers or belong to farming households. Sharmeen *et al.* (2010), and AK Narsani *et al.* (2012), reported 37.41% and 44.80% of the affected persons were farmers or agricultural workers.

About 65% of the patients were admitted of having taken corticosteroid eye drops before consulting proper physician. Adding another 15% patients with antibiotic eye drop use history it can be said that trauma might have deposited the fungal matter into non-intact cornea but it is the lowering of usual cell mediated immunity that increases the incidence of fungal corneal ulcer. Corticosteroid instillation is seen as the predisposing factor both in developed and developing countries with studies showing 8% (Srinivasan *et al.* 1997)[5] association.

Filamentous fungi are more common causes of corneal ulcer in tropical and subtropical climates. In our study 100% of the cases were caused by filamentous fungi. All of the culture positive cases had shown fungal hyphae in 10% KOH wet mount test in our study. Comparing other microscopy methods like 1% methylene blue mount, Gram staining with 52% and 35% positivity rates we have concluded that 10% KOH is the best and least cumbersome method. PA Thomas 2003 had given the sensitivity of KOH to be 75-90%.

Positivity of KOH mount method increases with time. Before declaring the scrape material negative for fungal element it should be kept in wet chamber for a minimum of 8-12 hours. This will also increase specificity as tissue and other debris will be digested by 10% KOH. Freshly prepared KOH solution is always more helpful as it lacks any debris. Increasing the concentration of KOH is not always helpful but may reduce the waiting time. KOH is an irritant so protective glove should be worn while handling.

Most commonly used Gram stain had a sensitivity of only 46%. In the review done by PA Thomas in 2003 Gram stain had shown to have a sensitivity of 45-73%. It is more helpful for bacterial causes than fungal.

Culture had been the gold standard of diagnosis of fungal corneal ulcer. But there are some drawbacks to

culture diagnostic methods. Fungi being ubiquitous in presence; a single colony can be considered as contamination. To make culture more specific inoculating more than one media plates or inoculating in a specific pattern would be more useful.

Fusarium spp (34%) has been the most isolated filamentous fungi in our study. Together with *Aspergillus flavus* they constitute nearly 64% of the case population. Most of the studies on fungal keratitis across the globe have identified and reported both *Fusaria* and *Aspergilli*, or one of these two genera as the predominant fungal taxa causing human keratitis (Chowdhary et al. 2005,[6] Gopinathan et al. 2009).[7] Without using molecular technology identification of the filamentous fungi to the species level is not always possible. *A. flavus* is also an important cause of keratitis and is reported in some studies to be the most frequent *Aspergillus* species causing keratitis (Thomas *et al.,* 1986).[8] We isolated *Curvularia species* in 3(13%) cases as the causative filamentous fungi. Mascarenhas J *et al.*(2013) of India also had shown occurrence of *Curvularia species* in 11% cases.

In our study we took the commonly used antifungals for susceptibility testing. Discussing the uncommon first in DD testing Voriconazole, Fluconazole and Ketoconazole were active against *Alternaria* and *Paecilomyces species*. *Amphotericin B* was ineffective against *Paecilomyces* but was able to prevent *Alternaria species*. *Itraconazole* gave intermediate action against *Paecilomyces spp.* whereas it has good activity against *Alternaria spp.*

When MIC was done for this species *Paecilomyces lilacinous* identified in the study was found to be susceptible to *Itraconazole* with MIC 1µg/ml but was found to be resistant to all other antifungals tested. Studies done previously with *Paeilomycesspecies* has given MIC for *Itraconazole* 8µg/ml and for *AmphotericinB* as >16µg/ml (Manuel Cuenca-Estrella, *et al.,* 2006).[9] In our study we also found MIC for Amphotericin B >16µg/ml.

With DD sensitivity testing we found Fluconazole sensitive against 10(43%) and resistant against 10(43%) and intermediately active against 14% cases. Nearly 57.1% of *Aspergillus spp.* and 75% *Fusariumspp.* were resistant to *Fluconazole*. One isolate of *Curvularia* was resistant to *Fluconazole*.

Aspergillus spp. is ubiquitously present in the environment. It penetrates already injured cornea (mostly injury due to vegetative matter) and establishes infection when the host immunity is further lowered (corticosteroid eye drops). All the *Aspergillus spp.* isolates of our study were associated with vegetative matter injury. All these isolates were highly susceptible to *Voriconazole* and *Itraconazole*. So the first line of treatment should be *Voriconazole* eye drops for *Aspergillusspp.* causing keratitis.

Conclusion

Fungal corneal ulcer is more common in rural areas with people involved in farming. Topical steroid along with trauma is most common risk factor. Filamentous fungi are most common aetiology. Azoles are commonly used drugs to treat fungal keratitis, however resistance to these drugs are now emerging. Identification of the fungus and its susceptibility pattern is the key to success in the management of fungal keratitis.

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