MYCOLOGICAL PROFILE AND ANTIFUNGAL SENSITIVITY OF INFECTIVE KERATITIS IN A TERTIARY CARE HOSPITAL OF SOUTHERN ODISHA

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ABSTRACT

Background: Infective keratitis is the second major cause of blindness next to cataract. Mycotic keratitis is an important ophthalmologic problem especially in developing countries including India. Fungal infection involving cornea is a fatal condition which needs early diagnosis and treatment to save the patient's eye. Though studies on mycotic keratitis have been reported from different part of India, to the best of our knowledge this study showing antifungal susceptibility is the first to be reported from Southern Odisha. **Objective:** The purpose of this study was to study epidemiological characteristics, predisposing factors, fungal etiology and antifungal susceptibility of common fungal isolates in infective keratitis cases. **Materials and Methods:** A prospective study was conducted from November 2015 to October 2017 in the Department of Microbiology and Ophthalmology M.K.C.G Medical College and Hospital .Relevant information was recorded using standard proforma of keratitis cases. Corneal scrapings were collected under strict aseptic conditions and subjected to10% KOH mount, Gram stain and culture. Identification of fungalagents were done as per standard microbiological procedures. An antifungal sensitivity test was done by microbroth dilutions as per CLSI reference method. **Results:** Over a period of two years 149 patients of infective keratitis were evaluated. Microbiological diagnosis of mycotic keratitis was established in 39 (26.17%) cases. Filamentous fungi were isolated more often than yeasts. The most frequently encountered filamentous fungi and yeasts were *Aspergillus* spp. 14 (35.89%) and *Candida albicans* 7 (17.94%) respectively. Males were more commonly affected and were mostly in the age group of 46-60 years. Ocular trauma due to vegetative matter was the most common predisposing factor. Natamycin was the most effective antifungal against filamentous fungi and amphotericin B was most effective for *Candida albicans*. **Conclusion:** Because of serious consequences of mycotic keratitis, it is very important to know the exact etiological agents and effective antifungals to save the eye of the patients. So laboratory confirmation should be undertaken and fungal infection should be ruled out before prescribing antimicrobial agents.

Keywords: Mycotic keratitis; Keratomycosis; Antifungal susceptibility.

INTRODUCTION

Keratitis is an inflammation of the cornea produced by infectious organism or non-infectious agents or stimuli. Infectious keratitis (microbial keratitis) is a potentially vision threatening condition that can be caused by bacteria, viruses, fungi or parasites.[1] Mycotic keratitis (keratomycosis) is an invasive infection of corneal stroma caused by variety of fungal species, that leads to inflammation and ulceration of the cornea. Fungal etiological agents of keratomycosis belongs to at least 70 species from 40 genera of filamentous fungi and yeasts. Of these majority of cases were caused by *Aspergillus*, *Fusarium* and dematiaceous fungal species.[2]

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Keratomycosis occurs all over the world but the incidence is highest in warm and humid climates and particularly in a rural environment. India being a tropical agricultural country has a higher prevalence of fungal keratitis.[3]Most common predisposing factor for keratomycosis seems to be the corneal trauma by vegetative or soil matter.[4]Other risk factors include an immune-compromised host, topical or systemic corticosteroid administration, dry eye, contact lens wearers and neurotropic cornea.

A study in India revealed that around1 % of the population or 9 million people will acquire microbial keratitis in each 10 years and 50% of these cases will be fungal.[5] The incidence of fungal keratitis has been reported to range between 25.6% - 36.7% in various parts of India.[6] Study conducted from South India reported that 44% of all central corneal ulcers were caused by fungi.[7]

Odisha being in subtropical region has favorable environment for fungal growth as majority of people are involved in agriculture and this makes them more vul-

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nerable to mycotic keratitis. Mycotic keratitis always remains a diagnostic and therapeutic challenge to Ophthalmologists. The major difficulties are related to establishing a clinical diagnosis, isolating the causative agents and treating the keratitis effectively with topical antifungal agents. Yet there has not been much study about keratomycosis in this part of Odisha. Thus this work would definitely give an idea regarding prevailing fungal agents and effective antifungals to be used in keratomycosis.

Objective: The purpose of this study was to evaluate epidemiological characteristics, predisposing factors, isolate and identify the etiological agents of mycotic keratitis with antifungal susceptibility of most common fungal isolated agents at a tertiary care hospital in the Southern Odisha.

MATERIALS AND METHODS

Study design: This prospective, descriptive study.

Ethics approval: The study was approved by the institutional ethical committee and was conducted after obtaining informed consents of the patients.

Study location: Department of Microbiology, M.K.C.G. Medical College, Berhampur with active collaboration of the Department of Ophthalmology

Study period: November 2015 to October 2017.

Sample size: 149 patients with infective keratitis

Inclusion criteria: A total of 149 patients with infective keratitis attending the Ophthalmology OPD/ IPD during the study period of all age groups were included in our study.

Exclusion criteria: Viral ulcers, neurotropic ulcers, healing ulcers and ulcers resulting from autoimmune disorders were excluded.

Methodology: Relevant information about sociodemographic data including name, age, sex, occupation, risk factors, duration of symptoms, self-medication was recorded using standard proforma. All patients were examined under slit-lamp bimicroscope by an Ophthalmologist. Under strict aseptic conditions corneal scrapings were collected by the Ophthalmologist using a sterile # 15 Bard-parker surgical blade, after instillation of local anaesthetic i.e. 0.5% proparacaine without preservatives.[8]The materials were collected both from the base as well as from the edge of the ulcer. Scrapings were taken several times to obtain as much as materials possible.

The material obtained was directly inoculated into solid media (10% sheep blood agar, 2 sets of Sabouraud's dextrose agar, chocolate agar) in a row of C-shaped streaks and also inoculated into the liquid media such as brain heart infusion broth. The material wasalso smeared on a labelled slide for 10% KOH wet mount and Gram's staining for demonstration of hyphae, pseudohyphae and yeastcells.[9]The culture plates (10% sheep blood agar, chocolate agar, BHI broth) were incubated for 48 hours at 37^{o}C , and was discarded if no growth was obtained and no turbidity was seen after 48 hours. The SDA plates were incubated at 37^{o}C and 25^{o}C separately. The SDA plates were examined daily for the 1st week, twice a week for subsequent period and upto 4weeks before discarding as negative. [3] Growth of mould on SDA was furthered identified by colony morphology, rate of growth, microscopic appearances in LPCB mount with slide cultures while yeast isolates were identified by standard tests like germ tube, chlamydospores production on cornmeal agar.[3]

The results were considered positive when smear results were consistent with culture and/or growth of the organism was demonstrated on two or more media and/or same organism was grown from repeated scrapings and/or growth was consistent with clinical signs. [10]

Antifungal susceptibility testing was done by microbroth dilution method as per CLSI reference method for broth dilution of antifungal susceptibility testing of filamentous fungi (M38-A2) and for yeast (M27-A3). The drug concentrations to be tested ranges may be; amphotericin B, ketoconazole from 0.0313-16µg/ml, for natamycin 0.313-32 µg/ml and for fluconazole 0.125-64 µg/ml.[11,12]

The culture medium used for this method was RPMI-1640 with glutamine without bicarbonate with pH indicator buffered with MOPS. Preparation of stock solutions and serial dilutions of antifungal agents were made. For water insoluble antifungal agents, dilution series were prepared first at 100X final strength in DMSO and each of them diluted tenfold in RPMI-1640 medium. Inoculum preparation of filamentous fungi was done by taking loopful growth from potato dextrose agar in 085% normal saline and then conidial suspension was adjusted to an optical density at 530 nm as per different fungi. Similarly yeast suspension prepared from taking colonies from fresh SDA agar plate and was matched against 0.5 McFarland or at 530 nm by photo spectrometer. Inoculation of micro titre plate was done by adding 100µl of fungal inoculum with 100µl of antifungal concentration with growth control and medium control. The microtitre plate was covered with lid and incubated at 35^{o}C for a duration given for various fungal species and MICs were determined as per CLSI documents.

All culture media and antifungals were obtained from Hi-media Laboratories, Mumbai, India.

RESULTS

The present study was conducted by taking 149 infectious keratitis cases in our department from November 2015 to October 2017. The various findings were observed and result was analysed as follows:

Table 1. Demographic characteristics of patients. (n=149)

DEMOGRAPHICS	PARTICULARS	n (%)
Sex	Male	95 (63.75)
	Female	54 (36.24)
Age (years)	0-14 years	3 (2.01)
	15-30 years	21 (14.09)
	31-45 years	45 (30.20)
	46-60 years	67 (44.96)
	>60 years	13 (8.72)
Residence	Rural	105 (70.46)
	Urban	44 (29.53)
Occupation	Agricultural workers	77 (51.67)
	Manual labourers	28 (18.79)
	Households	17 (11.40)
	Carpenters/welders	11 (7.38)
	Professionals	9 (6.04)
	Students	4 (2.68)
	Others	3 (2.01)
Seasonal variation	November-February	59 (39.59)
	March-June	43 (28.85)
	July-October	47 (31.54)

A total of 149 cases with clinical diagnosis of infective keratitis were studied for a period of 24 months. Out of total 149 cases, males were predominant 95 (63.75%) cases than females 54 (36.24%) with male to female ratio being 1.75: 1. Maximum number of patients were in the age group 46-60 years, 67 (44.96%) cases followed by 31-45 years which accounted for 45 (30.20%) of cases. Majority of cases were from rural areas 105 (70.46%) while only 44(29.53%) were from urban areas. Highest numbers of patients were agricultural workers 77(51.67%), usually working in paddy field followed by manual labourers in 28 (18.79%) cases. There was significant increase in patients during the month of November to February accounting for 59 (39.59%) cases. [Table-1]

The major predisposing factor of patients were found to be ocular trauma, in 87 (58.38%) cases followed by steroid eye drop use in 21 (14.09%) cases. Among the ocular trauma, vegetative injuries (leaf/stalk/grass) was the most common traumatic agents found in 39 (26.17%) cases. Other significant agents of trauma were sand/mud/dust, wooden objects, cow tail, insects and finger nail injuries. In addition, systemic diseases like diabetes mellitus, and HIV accounted for 15 (10.06%) cases. Ocular diseases predisposing to infective keratitis was present in 11 (7.38%) cases with 3 (2.01%) cases being soft contact lens users. [Table-2]

Table 2. Predisposing factors in patients. (n=149)

Predisposing factor	No. of cases	Percentage
Trauma	**87**	**58.38**
vegetative injury	39	26.17
sand/mud/dust	21	14.09
wooden object	12	8.05
Cow tail	5	3.35
Insects	3	2.01
Finger nail	2	1.34
Unknown foreign body	5	3.35
Ocular diseases	**11**	**7.38**
Conjunctivitis	5	3.35
Dacrocystitis	4	2.68
Trichiasis	1	0.67
Lagopthalmus	1	0.67
Systemic diseases	**15**	**10.06**
DM	11	7.38
HIV	4	2.68
Steroid eye drop use	**21**	**14.09**
Impacted corneal FB	**07**	**4.69**
Contact lens users	**03**	**2.01**
Unknown	**05**	**3.35**
Total	**149**	**100**

Table 3. Correlation between direct microscopic (10% KOH wet mount and Gram stained smear) diagnosis and cultures based diagnosis.

Investigation	Results	Presence of fungal growth in culture		Total	Sensitivity (%)	Specificity (%)
		Positive	Negative			
Detection of fungal elements in KOH mount	Positive	34	9	43	87.17	91.81
	Negative	5	101	106		
	Total	39	110	149		
Detection of fungal elements in Gram stained smear	Positive	13	6	18	33.33	95.45
	Negative	26	105	131		
	Total	39	110	149		

Table 4. Fungal isolates from patients of infective keratitis. (n=39)

Fungi	Pure fungal isolates (no.)	Mixed with bacteria (no.)	Total (%)
Filamentous fungi			**29 (74.35)**
Aspergillus flavus	5	3	8 (20.51)
Aspergillus fumigatus	3	0	3 (7.69)
Aspergillus niger	2	1	3 (7.69)
Fusarium spp.	4	2	6 (15.38)
Curvularia spp.	4	0	4 (10.25)
Acremonium spp.	0	2	2 (5.12)
S .apiospermum	1	0	1 (2.56)
Cladosporium spp.	1	0	1 (2.56)
Exserohilum spp.	1	0	1 (2.56)
Yeast			**10 (25.64)**
Candida albicans	5	2	7 (17.94)
Candida parapsilopsis	1	1	2 (5.12)
Candida tropicalis	1	0	1 (2.56)
Total	**28**	**11**	**39 (100)**

Table 5. In vitro antifungal susceptibility of commonly isolated fungus by CLSI reference method.

Isolated fungi (n)	Amphotericin B		Ketoconazole		Fluconazole		Natamycin	
	Range of MIC (µg/ml)	Mean MIC (µg/ml)	Range of MIC (µg/ml)	Mean MIC (µg/ml)	Range of MIC (µg/ml)	Mean MIC (µg/ml)	Range of MIC (µg/ml)	Mean MIC (µg/ml)
***A. flavus* (8)**	2-8	4.25	2-8	3.75	2-16	6.0	2-16	7.50
***A. fumigatus* (3)**	0.5-2	1.50	0.5-4	2.16	1-4	2.33	1-2	1.33
***A. niger* (3)**	1-4	3.0	2-8	4.0	2-8	4.66	2.0	2.0
***Fusarium* spp. (6)**	2-8	4.0	0.5-8	4.91	4-16	7.33	2-4	2.66
C. albicans (7)	0.5-1	0.57	0.5-4	2.21	1-8	3.57	0.5-4	1.42

Sensitivity and specificity of corneal scrapings to detect fungal elements in KOH wet mount examination with culture as gold standard were 87.17% and 91.81% respectively and that of Gram stained smear was found to be 33.33% and 95.45% respectively. Thus the sensitivity of 10% KOH wet mount preparation was higher (87.17%) than Gram stained smear (33.33%) in the detection of fungal elements. [Table-3]

Out of 39 fungal isolates (28 cases pure fungal isolates and 11 cases mixed with bacteria) 29(74.35%) were filamentous fungi and remaining 10 (25.64%) cases were yeast like fungi. Among molds the most common isolate was *Aspergillus* spp. in 14 number of cases (8; 20.51% *Aspergillus flavus*, 3; 7.69% *Aspergillus fumigatus* , 3; 7.69% *Aspergillus niger*) followed *by Fusarium* spp. in 6(15.38%) cases. *Candida albicans*in 7 (17.94%) cases was the most common yeast isolates followed by *Candida parapsilosis* in 2 (5.12%) cases. [Table-4]

Owing to a small sample size in various groups of fungi, the mean MIC was considered for comparison rather than MIC90 or MIC50. Antifungal susceptibility testing of most common fungal isolates showed that overall Natamycin was the most effective antifungal drug for filamentous fungi. The mean MIC of natamycin for *Aspergillus flavus* 7.50 µg/ml, *Aspergillus fumigatus* 1.33 µg/ml , *Aspergillus niger* 2.0 µg/ml, *Fusarium* spp 2.66 µg/ml. For *Candida albicans* MIC of amphotericin B was 0.57 µg/ml which is the lowest among the antifungal used, so it was the most effective antifungals to *C.albicans*. [Table-5]

DISCUSSION

Microbial keratitis is one of the major causes of preventable form of corneal blindness and mycotic keratitis forms an important component of the disease spectrum. There is an increase trend of fungal keratitis because of injudicious use of broad spectrum antibiotics, immunosuppressive drugs, corticosteroids and improved microbiological techniques. Mycotic keratitis is more commonly seen in tropical and subtropical regions than in temperate regions.[10] Odisha being in

the tropics with a climate and humidity that suits best for the fungal growth.

A total of 149 infective keratitis cases were included in the study. Demographics data showed that males were predominant in 95(63.75%) cases than females with male to female ratio 1.75: 1. Most common age group being 46-60 years followed by 31-45 years accounting for 67 (44.96%) cases. This is similar with the study conducted by Saha, et al.[8] , Chander, et al. [13], Routaraya, et al.[14] , who showed males predominance with common age group being 50-60 years. This is explained by the fact that, males are more involved in outdoor works with this age group being most socially active and working community of society, therefore at highest risk of getting the infection. Majority of people were from rural area 105 (70.46%) cases while only 44 (29.53%) were from urban area similar to Bharathi, et al.[10] Regarding occupation, 77(51.6%) of the patients were agricultural workers followed by manual labourers matching with study in South India and West Bengal where agricultural workers accounting for 65% and 57.7% respectively.[10,15]

In the present study, the maximum number of cases were found during November to February which accounted for 59(39.59%) cases corresponding to dry, chilly and winter season where harvesting were done in this region. Similar findings was observed in Hyderabad by Gopinathan U, et al.[16] Corneal trauma was most common predisposing factors in 87(58.38%) cases coinciding with other studies like Bharathi, et al., Saha et al., Despande, et al.[17] constituting about 92%, 48%, 55% respectively. Vegetative matter (leaf/stalk/grass) was observed as the most common traumatic agents (26.17%) followed by sand/mud/dust (14.09%) and wooden objects in (8.05%) cases.

Direct microscopic examination of corneal scrapings was found to be an important technique for etiological diagnosis. KOH wet mount examination was found to be very useful in early diagnosis of fungal infection. Sensitivity and specificity of corneal scrapings to detect fungal elements in KOH wet mount examination with culture as gold standard were 87.17% and 91.81% respectively and that of Gram stained smear was found to be 33.33% and 95.45% respectively. The sensitivity and specificity in various studies across the country shows a variable pattern but our findings correlates with the findings of study in South India where sensitivity and specificity of KOH wet mount as 99.23% and 99.06% and for Gram stained smear the sensitivity and specificity are 88.73% and 100% respectively.[10]

The prevalence of mycotic keratitis in this study was 39 (26.17%) cases. Similar reported findings in other regions of India are 7.3% in North India,[13] 22.25% in New Delhi,[9] 36.3% in Mumbai,[17] 25.6% in West Bengal[8] and 36.7% in South India. [10] This regional variation could be because of fungal keratitis is expected to be more common in tropical and subtropical regions than temperate regions. As shown by other studies, filamentous fungi were most commonly isolated than yeasts in our study (29; 74.35% versus 10; 25.64%). Among the filamentous fungi *Aspergillus* spp. 14 (35.89%)followed by *Fusarium* spp. 6 (15.4%) were the most frequently isolated fungal pathogens. Our results correlates with several different studies where *Aspergillus* spp. followed by *Fusarium* spp. were most common isolates of mycotic keratitis.[13,14,18] Among the *Aspergillus* spp., *Aspergillus flavus* was the most common fungal isolates in 8 (20.51%) cases. However , *Fusarium* spp. was more common fungal isolates as shown by studies from South India.[3,10] Other filamentous fungi isolated were *Curvularia* spp., *Acremonium* spp., *Scedosporium apiospermum*, *Exserohillum* spp. and *Cladosporium* spp. *Candida albicans* was the predominant yeast isolates in 7 (17.9%) cases followed by *Candida parapsilosis* 2 (5.12%) and *Candida tropicalis* in 1 (2.56%)cases.

Antifungal susceptibility testing against most common fungal isolates were done by microbroth dilution taking water insoluble antifungals like natamycin, amphotericin B, ketoconazole and water soluble antifungals like fluconazole. It shows that overall natamycin was the most effective antifungal drug for filamentous fungi. The mean MIC of natamycin for *Aspergillus flavus* 7.50 µg/ml, *Aspergillus fumigatus* 1.33 µg/ml, *Aspergillus niger* 2.0 µg/ml, *Fusarium* spp 2.66 µg/ml. For *Candida albicans,* MIC of amphotericin B was 0.57 µg/ml which is the lowest among the antifungal used, so it was the most effective antifungals against *C. albicans*. similar results of natamycin were obtained by Pradhan, et al, who showed natamycin was more effective against filamentous fungi because of low MIC except *Aspergillus flavus*.[19] A study showed that, amphotericin B is sensitive to all *Candida albicans* isolates from keratitis cases.[20]

CONCLUSION

This study highlights the relative prevalence of mycotic keratitis in this region. Fungal infection of cornea continues to be an important cause of ocular morbidity, mostly in males inhabiting rural areas, involved in outdoor agricultural activity. The key element is the clinical suspicion by the Ophthalmologist and laboratory confirmation of fungus before prescribing broad spectrum antibiotics/antifungals/corticosteroids. So proper identification and quick administration of appropriate antifungals therapy is the need of the hour for saving the eye and to prevent the catastrophe of life long blindness.

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