Fungal infections of the eye - laboratory diagnosis and treatment

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ABSTRACT

Infections of the eye give rise to severe ocular morbidity and blindness include keratitis, orbital cellulites, endophthalmitis and dacryocystitis. Corneal blindness, in developing countries is predominantly associated with infections. In India, nearly 30-35% of all culture positive infectious keratitis are caused fungi. Laboratory diagnosis mainly depends upon proper collection and transport of clinical specimens. In fungal keratitis, corneal scraping is the ideal sample, but occasionally corneal biopsy or anterior chamber aspirate may also be needed. Corneal scraping is usually by Kimura spatula, under a slit lamp examination, after anaesthetizing the cornea with topical anaesthetic like 0.4% proparcaine. Corneal biopsy is done by a minor trephining and AC aspirate using a sterile tuberculin syringe. In case of endophthalmitis, 150-200ìl of aqueous humour is collected. Vitreous fluid (500-1000 ìl), however, is collected by pars plana vitrectomy onto sterile tuberculin syringe, the needle is then fixed to a sterile rubber bung after expelling air from the syringe. The collected sample is immediately transported to the laboratory. Swabs from the regurgitating lacrimnal sacs and wound aspirate/ swabs are the ideal specimens for dacryocystitis and orbital cellulites, respectively. These samples are cultured onto SDA slants following standard procedures. The main draw back of culture is its long incubation time (5 to 14 days), though it is indispensable from the view point of the specificity. Direct examination (KOH wet mount, Gram's, Giemsa or calcofluor fluorescent staining methods) of the specimen, however, is quick and immensely helpful for ophthalmologist. The newer rapid methods, such as molecular techniques are also available and the management of patients can be according to the results obtained. With the advent of novel antifungal agents such as newer azoles and cell wall acting antifungals like echinocandins, the clinician has the wider option of selecting the therapeutic modality. In the event of the increasing reports of in vitro drug resistance to much frequently used azoles, polyenes and 5-fluorocytosines, clinical applicability of the newer antifungal agents seems to be quite promising.

Keywords: Fungal eye infections, orbital cellulites, dacryocystitis, endophthalmitis, keratitis, antifungal agents.

Fungal infections of the eye are important amongst the clinical conditions responsible for ocular morbidity and blindness. In tropical countries, including India, keratitis is the most frequently encountered fungal infection,[1] although the orbit, lids, lacrimal apparatus, sclera, conjunctiva and intra-ocular structures may also be involved. In the present review, discussion will mainly be focused on the most important clinical entities like (a) keratomycosis, (b) endophthalmitis, (c) orbital cellulitis with a very brief overview of dacryocystitis**.**

KERATOMYCOSIS

Fungal infection of the cornea (keratomycosis, mycotic keratitis or fungal keratitis) was described for the first time by Leber[2] in Germany in the year 1879. Since then it has been recognised as a major public health problem in the tropical parts of many developing nations including India.[3-5] Corneal infection of fungal etiology may represent 40-50% of all cases of culture proven infectious keratitis.[3,6] If not treated early, this condition may lead to corneal blindness. In contrast to this, the figures in western countries are variable. Whereas Keyhani *et al*[7] reported 0.6% of fungal infection following penetrating keratoplasty, Harris *et al*[8] reported 25% of keratitis due to *Candida albicans* following the same procedure.

Predisposing factors

Mycotic keratitis in the tropical and subtropical zones is largely due to filamentous fungi, although yeasts, particularly *Candida* may also be responsible in a small percentage of cases.[3] The most common predisposing factor is trauma to the eye with vegetative matter. In such cases, the history elicited from the patients is quite suggestive of fungal infection. Usually a healthy young adult male engaged in agricultural or outdoor work often gets traumatized in the eye with some kind of vegetative matter and he develops an ulcerative lesion 10-15 days following injury**.** In a series of recently conducted studies it was observed that trauma could predispose to ulcerative keratitis in 23-55% of cases.[4,5,9-11]

Seasonal variations are known to affect the frequency of isolation and types of fungi.[12] Other conditions include prior administration of corticosteroids and long term use of antibiotics.[10,11] The use of hydrogel contact lenses has also been associated with fungal keratitis.[3] For keratitis caused by yeasts, however, three important risk factors such as chronic ocular surface disease, contact lens wear and use of topical steroids are associated.[14] Recent investigations suggested the association of contact lens solution in the induction of *Fusarium* keratitis.[13] Males are significantly more frequently affected than females,

presumably because of trauma encountered by them at a higher frequency during outdoor activities.[15] In addition, other less frequently associated conditions include previous episode of keratitis, surgery, malnutrition, dry eye and facial palsy.[8,9]

Common etiological agents

Many fungal genera have been implicated in keratomycosis, the most frequently isolated being, *Aspergillus*, *Fusarium, Curvularia, Helminthosporium, Alternaria, Penicillium* and *Bipolaris* species. According to the published literature,[8,9] more than 56 genera of fungi, comprising of over 100 species have been incriminated as the causative agents of mycotic keratitis. A careful review of the literature shows the role of various fungal isolates in causing keratomycosis throughout the world, with differing predominant risk factors (Table-1).[4,5,10,11,14.16-19]

Pathogenesis

Invariably, fungi are not able to penetrate the intact corneal epithelium, unless the eye is severely immunocompromised due to long term use of antibiotics or steroids or due to any other predisposing conditions.[20] Trauma facilitates the penetration of the fungal inoculum deep into the layers of the cornea, even up to the stroma. Fungi being ubiquitous, trauma due to vegetative or organic matter helps in the diposition of spores on the injured cornea which acts as a very good substrate for the spores to germinate and give rise to hyphae.[20,21] The hyphal forms being invasive, can traverse through the stroma and reach the Descemet's membrane producing descemetocele. Even infection can reach and penetrate Descemets without producing a descemetocle which involves loss of all stromal tissues. There can be perforation of the entire cornea and the organism can reach the anterior chamber, giving rise to hypopyon. At

Table-1: Major risk factors and etiological agents of mycotic keratitis

Risk Factors (% of total)	Isolates (% of total isolates)	Reference (place of study)
Trauma (35)	*Aspergillus* (25), *Fusarium* (6.4)	Ref. 4 (Sri Lanka)
Trauma (55)	*Aspergillus* spp. (39.5)	Ref. 10 (India)
Systemic illness (11.1)	*Fusarium* spp. (14)	
Previous eye surgery (18.5)	*Alternaria* spp. (10.2)	
Contact lenses (3)	*Curvularia* spp (7.4)	
Ocular Surface disorder (41.7)	*Penicillium* spp. 97)	Ref. 14 (Philadelphia)
Contact lens wear (29.2)	*Candida albicans* (45.8)	
Atopic disease 916.7)	*Fusarium* (25)	
Ocular trauma (0.3)		
Topical steroids (16.7)		
Trauma (44)		Ref. 11 (Florida)
Topical medication (13)	*Fusarium* spp (68)	
Diabetes (12)	*Candida* spp (14)	
Topical steroids (7)	*Curvularia* spp (9)	
Contact lens (6)	*Aspergillus* spp (4)	
Trauma (39.2)	*Paecilomyces* (3)	Ref. 5 (Ghana)
	Fusarium spp (52)	
	Aspergillus (15)	
Trauma (65.4)	*Cladosporium* (6)	Ref. 16 (India)
Corticosteroids (8)	*Fusarium* spp (43)	
Traditional eye remedies (37)	*Aspergillus* spp(15)	
Trauma (42)	*Curvularis* spp(3)	Ref. 17 (India)
Contact lens wear (25)	*Aspergillus* spp (41)	
Topical steroids (21)	*Curvularia* spp (29)	
Trauma (50)		Ref. 18 (Paraguay)
	Acremonium spp (40)	
Trauma (82.9)	*Fusarium* spp (15)	Ref. 19 (India)
Topical steroids (19.3)	*Aspergillus* spp (59.8)	
	Fusrium spp (21.2)	

Table-2: Antifungal regime for keratomycosis

Yeasts: *First choice*: Amphotericin B 0.15% eye drops; Fluconazole(0.5% drops, 200mg orally)	*Alternatives*: Flucytocine(1% drops,150mg/kg orally); Miconazole 1% drops,subcunjunctival/injection; Ketoconazole 1% drops; Ketoconazole 200mg BD orally
Filamentous fungi: *First choice*: Natamycin 5% drops	*Alternatives*: Amphotericin B 0.15% drops + FlucytosineItraconazole (1% cream + 200-400 mg orally)

times one can even have a hypopyon without fungal invasion of the anterior chamber.

Once, invasion occurs, the intrinsic virulence of fungi, helps them to proliferate within the corneal tissue, resisting the host defense and producing tissue damage.[2] The large sizes of the hyphae of filamentous fungi and the pseudohyphae of yeasts preclude complete ingestion by macrophages and neutrophils. Toxins and enzymes such as hemolysins, exotoxins and proteases, liberated by the fungi contribute to the tissue damage, accentuated further by the host inflammatory response.[22,23]

Clinical features

The onset is usually insidious, often following corneal injury. The ulcer often runs on indolent course. The epithelium may show defect at the site of infiltration or epithelial defect would have healed with deep stromal infiltrates.[24,25] Endothelial plaque with moderate degree of hypopyon may be noticed.

The ulcer has a raised, wet, soft, creamy to greyish white infiltrate. However, filamentous fungal infections are not very wet until the case is well advanced**.** The ulcer has feathery or hyphate margins and satellite lesions may also be seen.[24] The symptoms like pain, photophobia and redness are more severe as compared to those seen in bacterial keratitis. Features of keratitis due to *C. albicans* and other yeasts resemble bacterial keratitis with an overlying epithelial defect, a more descrete infiltrate and slow progression. Such ulcers frequently occur in eyes with preexisting corneal disease**.**[26]

Laboratory diagnosis

Once there is clinical suspicion of a fungal infection, every effort should be made to recover the causative fungus so that appropriate antifungal therapy may be instituted timely. The various clinical samples, for laboratory diagnosis, include (a) corneal scraping (b) corneal biopsy and (c) anterior chamber aspirate.

Corneal scraping

Scraping is collected after anaesthetising the cornea with 0.5% proparcaine drops and waiting for 2-3 minutes. With the help of sterile Kimura spatula or Bard-Parker blade No.15 or Iris repositor, scraping is done by applying multiple, moderately firm, unidirectional strokes, under slit lamp illumination. Material is collected both from the base as well as from the edge of the ulcer, after retracting the lids properly and after cleaning any discharge or debris from the vicinity of the ulcer. Collection of a mere corneal swab is not recommended. Use of a calcium alginate swab is sometimes advised for better yield of fungus.[27] However, its utility is still debatable.

Corneal biopsy

It is a relatively invasive (trephining) procedure and requires minor OT. The indications of biopsy are (a) strong clinical suspicion of fungal keratitis (b) atleast twice negative smear and culture report (c) no clinical improvement on empiric antibiotic therapy. The biopsied material is preferably removed enbloc. It is bisected, half being sent to microbiology laboratory for homogenization and culture and smear examination, and the remaining half put in 10% buffered formalin for histopathological examination.

Anterior chamber aspirate

Anterior chamber (AC) paracentesis is done when there is strong clinical suspicion of intra ocular infection. In addition, progressive corneal damage and persistent hypopyon are also indicative of this procedure. The aspirate is collected with the help of sterile tuberculin syringe and 22 gauge needle. The AC is tapped via the limbus. The needle should be removed before the specimen is submitted in order to decrease the danger to laboratory personnel. However, the nozzle of the syringe should be sealed with a sterile rubber bung and the whole set should be transported immediately to the laboratory for processing.

Processing of samples

As a routine, the scraped out corneal tissue or the biopsied material after homogenization is divided into three portions, one for Gram staining, one for 10% KOH wet mount and the third for culture. The reported rate of utility (sensitivity) of simple KOH wet mount for the presumptive diagnosis of fungal keratitis varies between 33 to 92%.[28,29]

Gram stain, though has been reported to yield an accuracy of 60-75% in detecting the causative organism,[30] is undoubtedly a simple and rapid method. A comparative evaluation on the efficacy of gram staining of corneal scraping with hematoxylene and eosin staining yielded a better diagnostic efficacy[22] as compared to gram staining and KOH wet mount.[31] Other staining techniques like periodic acid schiff (PAS) staining, Gomori's methenamine silver staining, calcofluor white, acridine orange, fluorescent stainings have also been recommended.[32]

***Culture and identification*:** Apart from the conventional culture techniques on SDA slants, and lactophenol cotton blue (LCB) preparation of the growth for distinguishing between yeasts and mycelia[33] and for the identification of mycelia fungi, one can also opt for a slide culture technique[33] which visualizes aerial hyphae of moulds making the microscopic identification easier. Yeasts can be speciated by looking for chlamydospore formation on cornmeal agar and germ tube production as well as various sugar fermentation and assimilation tests, urease test and other biochemical tests.[34]

***Interpretation of culture report*:** Fungal spores being ubiquitous, interpretation of fungal growth in the laboratory is sometimes difficult. Moreover, the causative agents of mycotic keratitis are often saprophytic. Therefore, in order to attribute clinical significance to a particular growth, the following criteria need to be considered (1) the laboratory finding should be correlated with clinical presentation, (2) inoculation should be done on 'C' streak manner and growth occurring only on the 'C' streak is considered significant, (3) smear results should be consistent with culture, (4) the same fungus should grow in more than one culture medium and (5) the same organism should grow from repeated scrapings.

***Molecular methods for the diagnosis of mycotic keratitis*:** Polymerase chain reaction (PCR) assay, PCR-SSCP (single stranded conformational polymorphism) and PCR-RFLP (restriction fragment length polymorphism) techniques have also been standardized for fungal identification.[35] Of these, the PCR is universally accepted as most popular technique as it can yield quick results, confirming the diagnosis of mycotic keratitis within a few hours, whereas culture takes at least 5 to 6 days for a positive detection.[36] In a recent study,[37] a PCR-based assay, developed to amplify a part of the fungal 18S r-RNA gene, was used for detection of fungal DNA in corneal scrapings. PCR and fungal culture results matched in 74% of cases. Thus, PCR assay, presently, seems quite promising for the diagnosis of fungal keratitis, offering definite advantage over culture methods. However, its main drawback is its occasional false positivity that can be overcome by application of stringency in laboratory procedures and proper standardisation of the techniques, PCR remains to be an effective method in diagnosing keratomycosis. It is also a more sensitive and rapid method than the conventional mycologic procedures. Besides, PCR is of great benefit in rapidly detecting the presence of the organism difficult to culture. The sensitivity of PCR, taking culture as the gold standard, was quite high between 89 to 94%, whereas, specificity ranged between 50% to 88%.[36,37]

Table-3: Bacteria and fungi isolated from 86 eyes with congenital dacryocystitis*

Bacteria		**Fungi**	
Staph. epidermidis	*28*	Candida albicans	5
Str. pneumoniae	*24*	Aspergillus niger	5
Staph. aureus	*6*	*Rhizopus* sp.	*3*
Ps. aeruginosa	*2*	Aspergillus flavus	2
Enter. aerogenes	*2*	*Penicillium* sp	*2*
Proetus mirabilis	*2*	*Alternaria* sp	*2*
Esch coli	*1*	*Phialophora* sp	*2*
Acinetoebacter sp.	*1*	*Trichoderma* sp	*2*
Sterile	*20*	*Drechslera* sp	*1*
		Curvularia sp	*1*
		Mixed	1

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Treatment of fungal keratitis

If direct microscopic examinations of corneal scrapes or corneal biopsies yield definite results that are consistent with the clinical picture, treatment should be initiated immediately.[3] The antifugnal agents available today to combat fungal keratitis are not so well developed as those available against bacterial infections. Most of the available agents only inhibit the growth of the fungus necessitating the host defense mechanisms to eradicate the infection.[38] The currently used antifungal agents belong to, (1) Polyenes, (2) Azoles, including newer azoles (3) Pyrimidines, (4) other derivatives

Polyenes: The polyene bind to the ergosterol of fungal cell membrane, creating pores that disrupt the homeostatic mechanisms leading to cell death. Nystatin was the first polyene antifungal to be identified. It has been recommended for topical use (100,000 units). However, corneal toxicity and poor ocular penetration limits its value.[38]

Amongst polyenes, Natamycin is often the first drug of choice for filamentous infections since it is easily available. It is marketed as a 5% suspension for topical use and has broad spectrum of activity. A 5% suspension in the eye is well tolerated. However, this drug may be ineffective in cases with deep stromal abscess because of poor corneal penetration.[38]

Amphotericin-B has widely been used as a topical and systemic drug for ocular infections. Preparation of a 0.15% suspension of Amphotericin B, reconstituted from the 50mg vial power (for IV formulation) is universally adopted for topical use as the first line drug both for *Candida* keratitis as well as for keratitis due to other mycelial fungi.[14] After topical application, this drug can penetrate deep into the corneal stroma and 0.15% suspension is well tolerated, when istilled round the

Table-4: Organisms isolated from congenital dacyocystitis (N=112)*

Bacterial	**48 (42.9%)**	**Fungal**	**6 (5.4%)**
Coagulase neg. Staphylococcus	24 (21.4%)	*Fusarium spp.*	2 (1.8%)
Str. pneumoniae	14 (12.5%)	*Asp. niger*	1 (0.9%)
Stpah. aureus	3 (2.7%)	*Helminthosporium* sp.	1 (0.9%)
Diphtheroids	3 (2.7%)	*Candida albicans*	2 (1.8%)
Pseudomonas sp.	2 (1.8%)	*Sterile*	106 (94.6%)
Streptococcus sp.	1 (0.9%)		
Proteus sp.	1 (0.9%)		
Sterile	64 (57.1%)		

**Reference No. 84*

clock every 15 to 30 minutes. However, intravenous administration, with the recommended dosage, may cause poor corneal bioavailabilitiy. The drug shows nephrotoxicity after systemic administration.

Fluorocytosine (Pyrimidines-5), a synthetic pyrimidine analogue, is available in the form of 1% suspension for topical use. It can be given orally (150mg/kg) as well. It has synergistic effect with Amphotericin B. Its topical form is nontoxic to the eye. If given systemically, it may cause transient bone marrow depression and gastrointestinal upset. The main drawback of this drug is its limited spectrum of activity against filamentous fungi and rapid development of resistance by *Candida* species.[39,40]

Azoles: These are the derivatives of imidazole ring with substitution mainly in position 2. The imidazoles and the structurally related N-substituted triazoles are considered together because they share the same antifungal spectrum and similar mechanism of action. However, systemic triazoles have a longer half-life than imidazoles. The imidazoles include clotrimazole, miconazole and ketoconazole. The triazoles include fluconazole and itraconazole.

Clotrimazole is usually used topically for skin and genital *Candida* infections. It is marketed as a 1% lotion for fungal dermatitis and as 1% cream for candida vaginitis. A 1% vaginal cream placed into an ophthalmic ointment container can be used for topical use in the eye for the treatment of keratomycosis.

Miconazole can be used as 1% eye drop, or through systemic infusion (20mg/kg body weight).[41] The 1% topical application is well tolerated and is reported to be quite successful in treating keratomycosis due to *Aspergillus* and *Candida* species[42]. A perspective series from India[43] found that it was effective in 64.7% of the cases when administered topically every 2 hours. It is usually reserved as a second-line drug in the management of fungal keratitis.[2]

Ketoconazole is another imidazole with pharmacological properties similar to that of miconazole; however, it is less toxic and absorbable from the gastro-intestinal tract. It is marketed as oral preparation in the form of 200mg tablets. It can also be administered topically as 2% eye drops. In earlier studies conducted on a rabbit models[44,45] of keratitis it was found that there was effective intra-ocular penetration of ketoconazole after oral administration. It was also reported in the literature that this drug was quite effective both as a prophylactic and a therapeutic agent when administered topically in an *A. flavus* model of keratitis.[46]

Fluconazole is a triazole compound, which can be administered both systemically (200-400mg per day) and topically (o.2% eye drops). After topical application, as 0.2% eye drop it shows good penetration into the anterior chamber.[47] The topical preparation is also well tolerated.

Itraconazole is a newer triazole which has larger spectrum of activity than fluconazole against filamentous fungi.[2] However, its only drawback is that it is quite hydrophobic, and being 90% protein-bound in the serum, does not penetrate the tissue as well as fluconazole does. A series from India reported an effectivity rate of 69% when topical or systemic itraconazole was used as the sole therapy for keratomycosis.[48]

Coad *et al*,[49] on the basis of tube dilution minimal inhibitory concentration and minimal fungicidal concentration testing determined that the imidazoles such as miconazole and ketoconazole consistantly showed the lowest Geometric mean titre for filamentus fungi. Thus, systemic azoles, in general have a good penetration and are frequently used for fungal keratitis. However, they have drug interactions and require monitoring of liver function tests.

Newer azoles: Voriconazole is a new azole with broad spectrum efficacy for fungal keratitis and endophthalmitis. In a recent study,[50] invitro susceptibility of various fungal isolates in infectious keratitis towards Voriconazole was 100%, Ketoconazole 82.4%, Amphotericin-B 76.5%, Itraconazole 67%, Fluconazole 60% and 5-Fluorocytocin 60%. Voriconazole MIC (90) was lowest for *Candida* species (0.016 mg/ml). They concluded that voriconazole was a better alternative for the therapeutic management of *Candida* and *Aspergilus* ocular infections, as compared to other antifungal**.**

Topical voriconazole was tested in rabbit models of fungal keratitis. Sponsel *et al*,[51] tested topical voriconazole in such a model of *Paecilomyces* induced keratitis. It was their observation that voriconazole therapy caused lesions to decrease within 8 days. Hyphal masses were present in the control infected eyes (not treated with the drug), but absent in the treated infected

eyes (as observed after sacrificing the animals and examining the sections of the eye ball). In their opinion, topical voriconazole is a good and effective alternative to topical Amphotericin-B, because *Paecilomyces* species are often resistant to Amphotericin B.

In yet another development, Ozbek *et al*,[52] emphasised the role of voriconazole in the management of *Alternaria* keratiits.In this study, they reported a case of a 69 year old man with a history of corneal foreign body removal, who developed a stromal infiltrate two months later. The condition did not improve despite topical antibiotics and natamycin. Repeat culture revealed *Alternaria* species, and topical Amphotericin-B was started. When there was no response, treatment was switched over to oral and topical voriconazole. Steady resolution was noted within ten days of therapy. Thus, they advocated that, voniconazole provided clinical improvement of keratitis due to *Alternatia*, which was earlier unresponsive to Amphotericin B.

Other derivatives: Echinocandins: These drugs have recently emerged as valuable antifungal agents. These are cell wall acting agents unlike Amphotericin-B which acts on fungal cell membranes. These drugs inhibit B 1-3 glucan synthesis, and include Caspofungin and Micafungin. Recently, topical Caspofungin was tried in a rabbit model[53], in which a 0.5% suspension of the drug was found to be as effective as 0.15% Amphotericin-B, in the treatment of keratitis caused by *Candida*.

Povidone-iodine (Betadine) and Polyhexamethyl biguanide (PHMB): The effectiveness of Povodone-iodine and PHMB as topical antifungals was evaluated by a study group in India[54] in experimentally induced Aspergillus fumigatus keratitis in rabbits. Keratitis was induced by corneal intrastromal infection of spores of *A. fumigatus* in four groups of six healthy rabbits each. Drugs used were 5% Natamycin, 0.02% PHMB, 1% Betadine and 0.5% hydroxypropyl methyl celllulose (HPMC) as control. The average healing time of ulcers were 21.5 ±3.08 days for Natamycin, 27.8±2.28 days for PHMB, 36.4±2.57days for Betadine and 38.2±4.7 for HPMC. While no corneal perforation occurred with Natamycin therapy, there was one perforation case with PHMB, there with Betadine and 5 with HPMC. Thus, 1% Betadine was not effective in fungal keratitis while PHMB 0.02% was moderately effective**.**

Therefore, the overall view on the management of mycotic keratitis is that the condition responds slowly over a period of weeks to antifungal therapy. Thus, in order to evaluate the prognosis, clinical signs of improvement should carefully be noted; these include deminution of pain, decrease in the size of the infiltrate, disappearance of satellite lesions, rounding of the feathery margins of the ulcer and hyperplastic masses or fibrous sheets in the region of healing fungal lesions[3]. Negative scrapings during treatment do not always indicate that fungal infection has been eradicated, since there may be active proliferation of the fungi deep in the stroma; hence therapy should be continued for at least 6 weeks, depending upon the antifungal agent selected (Table-2).

Patients with deep stromal infections and those who have received corticosteroids appear to respond poorly to medical therapy.[55] Surgery may be necessary in such cases. Every attempt is made, however, to prolong medical therapy for as long as possible, since this renders the infecting fungus nonviable, thereby improving the outcome of surgery. At the same time surgery may help medical management by increasing drug penetration.For example, in small superficial corneal fungal infections, regular surgical debridemet of the base of the ulcer helps elimination of fungi and necrotic material, facilitating the penetration of antifungal drugs into the corneal stroma.[3]

Surgery also helps in supporting the globe, where integrity of the globe is threatened as in case of thinnng or perforation of the cornea. If there is persistent epithelial defect, the usual recommendtion is a superficial lamellar keratectomy with removal of necrotic stroma and placing a thin conjunctival flap over the ulcerated site.[3] Blood vessels present on the conjunctival flap help in rapid healing of the ulcer. Recently, Kim *et al*[56] reported amniotic membrane transplantation as a good alternative for successful healing of corneal ulcers refractory to medical management.

However, in situations where there is keratitis with deep stromal lesions or progressive keratitis with corneal perforation not responding to antifungal treatment, a penetrating keratoplasty[38] is recommended. In this procedure, at least 0.5mm of clear corneal tissue is excised all around the infected area in order to decrease the chances of recurrence**.**

The advantage of this procedure includes the elimination of fungi simultaneously with the secondary inflammatory reaction. Topical treatment with antimycotics is recommended for several days post- operatively. An alternative therapy may be the use of excimer laser at 193 nm.[57]

Influence of fungal species on clinical presentation, therapeutic management and outcome of infection

Although most cases of mycotic keratitis exhibit the basic clinical features enumerated earlier, there may be certain unique features, depending upon the aetiological agent. In general, keratitis caused by filamentous fungi may involve any part of the cornea with firm elevated slough, hyphate lines extending from the ulcer margin, granular infiltrates and satellite lesions.[3] An endothelial plaque and hypopyon may be seen 5 to 6 days.[55] However, considering particularly about certain common agents causing mycotic keratitis, one would surely appreciate

Table-5: Organisms isolated from regurgitated materials in acquired dacryocystitis (N=110)*

Bacteria	37 (33.5%)	Fungus	5 (4.5%)
Coagulase neg. Staphylococcus	19 (17.3%)	*Fusarium* sp.	2 (1.8%)
Str. pneumoniae	3 (2.7%)	*Asp. niger*	1 (0.9%)
Staph. aureus	5 (4.5%)	*Curvularia* sp.	1 (0.9%)
Diphtheroids	3 (2.7%)	*Alternaria* sp	1 (0.9%)
Pseudomonas sp.	3 (2.7%)	Sterile	105 (95.5%)
Streptococcus sp.	1 (0.9%)		
Proteus sp.	1 (0.9%)		
Alkaligenes sp.	1 (0.9%)		
Acinetobacter sp.	1 (0.9%)		
Sterile	73 (66.5%)		

**Reference No. 84*

that the most common fungi like *Fusarium* species produce very severe infection with rapid onset of perforation of the cornea. Vision may be completely lost if timely therapeutic intervention is not initiated.[58,59] The same is true for *Aspergillus flavus* infection. Both these agents produce toxins and extracellular enzymes like proteinases. Some studies[6,60] revealed that corneal infections due to *Aspergilli* and *Fusarium* species are so severe that, in addition to the signs and symptoms mentioned above, around 42-60% of those may lead to malignant glaucoma. In most of the cases the features is so severe, that therapeutic keratoplasty is often indicated. Infection due to certain dematiaceous fungi (*Curvularia* or *Bipolaris*) is presented with persistent, low grade, smouldering type of keratitis with minimal structural alterations. Not infrequently, the necrotic slough may be pigmented. However, complication like perforation is less likely unless the cases is properly managed or augmented by steroids.[55] *Pseudallescheria boydii* is another mycelial fungus which often gives rise to severe form of keratitis with very poor clinical improvement, in spite of all possible medical therapy and may thus require surgical intervention.[61,62] In contrast to the features of certain difficult filamentous fungal infections enumerated above, the stromal keratitis due to yeasts quite often resembles bacterial keratiits[3] and thus can usually be managed with recommended antifungals.

Considering the aforementioned clinical situations, therapy of such cases always remains a challenge before the treating Ophthalmologist[52] and thus, testing for antifungal drug susceptibility seems to be a suitable solution to this.[60,62]

However, there is always an ambiguity in the interpretation of antifungal susceptibility test results which shows variability from laboratory to laboratory. This is mainly due to lack of standardization of different variables of this test, such as incubation temperature, incubation time, inoculum size and composition of test media[63], and more so the establishment of the breakpoint value for antifungal resistance. As for example, the investigators in a recently conducted study[64] could not take the CLSI (Clinical and Laboratory Standards Institute) breakpoint values into consideration for the interpretation of EUCAST (European Committee on Antibiotic Susceptibility Testing) MIC data.

However, some progress has been made in this field during the past decade with the standardisation of various parameters of antifungal sensitivity testing. The methods currently recommended by CLSI (M38 A,M 27A documents) have been adopted by many laboratories for the standardization of the techniques both for filamentous fungi and yeasts and reproducibility of the results have been claimed.[63-66]

A recent study,based upon such testing, documented the superiority of Voriconazole over Itraconazole towards Fluconazole resistant *Candida* isolates. Thus, antifungal drug sensitivity testing in a routine laboratory could help the clinician in prescribing drugs which are effective against a particular clinical isolate, rather than putting the patient on empirical therapy without knowing whether the patient is going to respond to the prescribed treatment or not. In this context, the results of a recent study[67] are noteworthy. While studying on the risk factors and treatment outcome in fungal keratitis the authors highlighted the importance of selecting the appropriate antifungal agent particularly for patients who were refractory to the primary therapy.[67] In addition to this there are scanty reports of inadequetly treated fungal keratitis (becuause the sensitivity pattern was not known) leading to serious complications like endophthalmitis.[68] All the above mentioned observations only point towards one thing that antifungal susceptibility testing is a prerequisite in the management of all problematic clinical situations mentioned above.

ORBITAL CELLULITIS

Orbital cellulitis is an infection of the soft tissue surrounding the orbit. Orbital cellulitis of fungal origin is the most serious ocular infection with significant potential morbidity, including loss of vision, cavernous sinus thrombosis, intracranial spread of infection and occasionally death.[60,69] Therefore, it is essential that patients with peri-orbital infection need careful evaluation and treatment. For this, study of anatomy of the orbit and its adjascent structures and the pathophysiology is essential.

Anatomy of the orbit and pathophysiology

Several anatomic features include, the thin and compliant nature of the eyelid; the proximity of the orbit to the paranasal sinuses, nasolacrimal apparatus, and the teeth;

and haematogenous communications through a valveless venous system, between the orbit and the surrounding facial compartments, especially the paranasal sinuses.[70]

Pre-septal cellulitis is the term given to inflammation confined to the space anterior to the orbital septum. Orbital septum is an extension of the periorbita (periosteum of the orbital bone). The septum arises from the orbital rim and inserts into the lower border of the tarsal plate inferiorly and into the levator aponeurosis superiorly. Thus, the orbit is delimited anteriorly by the orbital septum, which is generally an effective barrier that prevents spread of infection from the eyelids into the orbit. Therefore, in pre-septal cellulitis, severe signs of orbital disease such as proptosis, ophthalmoplegia and visual loss are absent. Because of the loose and elastic connective tissue attachments between the eyelid skin and the underlying structures, the degree of swelling of the eyelids is quite remarkable.

In contrast to this, the picture in postseptal cellulits is different. The periosteum of the orbit (periorbita) is very tightly attached to the suture lines and the orbital rims, but more loosely adherent to the underlying bone elsewhere. For this reason, infection spreading directly from the sinuses to the orbit usually takes the form of a subperiosteal abscess. The pre-septal cellulitis usually occurs in the following manner[69,71] (1) secondary to localised infection or inflammation of the eyelids or adjascent structures including hordeola, accute chalazia, acute dacryocystitis, impetigo, herpetic blepharitis or severe conjunctivitis, (2) secondary to eyelid or facial trauma and (3) in patients with a recent history of upper respiratory tract infection. The mechanism of post-septal cellulitis is, however, totally different. The thin porous bony wall of the orbit is surrounded by the paranasal sinuses. Medially, the lamina papyracia of the ethmoidal bone is less than 0.5mm thick.[60] It is easily fractured in response to direct or indirect trauma, and, frequently there are congenital dehescenses in the bone, permitting communications between the ethmoidal sinus and the orbit. Infection may also spread from the ethmoidal sinuses to the orbit via foramina through which the ehmoidal arteries pass. Lastly, the venous drainage of the orbit occurs posteriorly via the superior and inferior ophthalmic veins, which traverse the superior orbital fissure and empty into the cavernous sinus. The ophthalmic and ethmoidal veins communicate directly. Because, these communicating veins are valveless, pressure from the sinuses due to any inflammatory process may cause retrograde blood flow into the veins of the orbit and peri-orbital structures, thus, allowing the sinus infection to spread haematogenously to the orbit.

Sometimes, in the upper eyelid, the separation between the insertion of the orbital septum and the upper border of the tarsus is an unprotected area that may occasionally permit a pre-septal infection to spread posteriorly into the orbit.

Etiological agents and their pathogenicity

The most important and life threatening clinical entity is acute rhino-cerebro-orbital-zygomycosis (RCOZ). *Mucor* species are more frequently involved than the other genera of the order Mocorles, (Rhizopus, Rhizomucor and Absidia).[72,73] Recent reports of the thermophilic fungus *Apophysomyces elegans,*[74] causing invasive fungal infection like orbital cellulitis, suggests that the property of angio-invasiveness of these fungi accounts for their pathogenicity. Besides, *Aspergilus* species like *A. flavus, A. niger, A. fumigatus* can also invade vascular structures and thus can devastate orbital structures by spreading from their primary site of infection i.e. sinus. In addition to the above mechanisms, *Mucorales* group of organisms, especially *Mucor* species produce ketoreductase enzyme, which helps them to thrive in the host, who is invariably diabetic and keto-acidotic.[73] Even neutrophil dysfunction induced by diabetic ketoacidosis, further accentuates the fungal pathogenicity.[75] Other putative host factors, which augment the spread of the pathogen and in the detorioration of the condition especially that of RCOZ include neutropenia during chemotherapy, immunosuppression (organ transplantation, prolonged corticosteroids therapy, hemodialysis, malnutrition, intra-venous drug abuse, leukaemia, aplastic anemia, myelodysplastic syndrome, severe burns and long term antibiotic use).[75]

Clinical features

Ocular and orbital involvement in RCOZ is a very destructive opportunistic infection with severe dreadful clinical outcome, fatality having been reported in majority of cases.[74] Most of the patients are diabetic with a recent episode of ketoacidosis, but the condition may even rarely affect patients with good control of blood sugar. Clinical conditions such as severe diarrhoea and renal failure, which often lead to severe metabolic acidosis can also predispose to such infection.[73] In rare instances, no underlying cause is found. All age groups are affected. Patients usually come with acute episode of nasal congestion, rhinitis and facial pain. All patients are febrile and have markedly elevated white blood cell count invariably exceeding 20,000/mm^3. The infection progresses very fast and the patient experiences headache, convulsions and ultimately becomes comatose. On examination, one finds necrosis and perforation of the hard palate and necrosis of the nasal mucosa with thick gangrenous tissue in the nasal cavity appearing as black eschar. Orbital invasion is heralded by intense pain. Apical involvement is common, causing proptosis, visual loss, ophthalmoplegia and sensory loss along the distribution of trigeminal nerve. A CT scan at this stage may delineate involvement of paranasal sinuses, which may guide the clinician in taking decision

regarding the steps of management. Whether any specific radiological findings are diagnostic of RCOZ is controversial, although CT non-enhancement of the superior ophthalmic artery and vein, which is related to vasculitis and thrombosis, may represent one such specific sign.[72-74] However, apart from the clinical parameters mentioned above, a prompt and accurate diagnosis of this condition necessitates a reliable and correct laboratory diagnosis.

Laboratory diagnosis

***Clinical samples*:** Important clinical samples to be included are (1) pus/aspirate (2) exudate (3) thick nasal discharge (4) black eschar from the perforating hard palate (5) orbitotomy tissue (6) biopsy from the necrosed tissue. Blood can also be collected for fungal culture.

***Processing, culture and identification*:** Samples should be transported to the laboratory without any delay. If delayed, samples should be refrigerated, preferably in Stuart's transport medium.[74] Blood sample is inoculated onto the biphasic medium of BHI agar with BHI broth overlay and incubated at 37^0C. But the blood culture bottle should not be refrigerated, even if there is delay in transportation. Tissue and biopsy samples should be minced, and not ground in order to avoid the destruction of any viable fungal elements.

All samples except the blood is subjected to Gram staining and 10% KOH wet mount for fungal hyphae. Demonstration of zygomycetes in the smear of the necrotic tissue or the thick black eschar from a symptomatic case is quite diagnostic.[75] Part of the sample can also be stained with hematoxylin-eosin, which serves as a good adjunct to Gram staining and KOH mount. For the isolation and identification of the fungal species, sample should be inoculated onto SDA slants, the rest of the procedure being the same as described for fungal keratitis above.[33] Blood culture bottles are incubated at 37^oC in inclined position (at an angle of 30^0) for 1 hour everyday to allow sub- culturing of the organism growing in the broth onto the agar slant (Castaneda method of blood culture). Growth of any kind should be identified according to the standard techniques.[33]

Treatment

Systemic Amphotericin-B (AB) is the drug of choice. As has been emphasised earlier, the condition being acute and fatal, clinical diagnosis and positive smear report are quite suggestive of starting of therapy without waiting for the culture report. Conventional AB being nephrotoxic, it is administered with gradual increase in the dose each day, starting with 0.25mg/kg/day, increasing each day by 0.25mg/kg/day till a dosage of 5 mg/kg/day is achieved. It should be given through slow intravenous drip in 5% dextrose saline with constant monitoring of blood urea. If there are features of renal toxicity, systemic AB should be stopped and one can switch over to oral itraconazole 200mg twice daily for 4-6 weeks. If no renal toxicity and the clinical condition of the patient improve with AB, then the patient is put on itraconazole therapy, AB being omitted. Attempts have also been made to deliver AB directly to the infected orbital tissue, in the form of daily irrigation and packing.[76]

In addition, several novel formulations, AB colloidal dispersion and liposomal Amphotericin-B[76,77] have also been tried with excellent results, keeping in view the potent toxicity of the conventional drug. However, controlled trials are needed to assess the efficacy of these lipid formulations and of the conventional AB in the therapy of ophthalmic mycoses.

Other therapeutic options

Earlier studies showed that 100% hyperbaric oxygen at 1 to 3 atms, exerts a fungistatic effect. Hyperbaric oxygen may decrease tissue hypoxia, enhance oxygen-dependent cidal mechanism, and decrease tissue acidosis.[76] Exposure to 100% oxygen at 2-2,5 atms for 90-120 minutes every 12 to 24 hours is supposed to be quite effective.[75,78]

Surgical debridement of the necrotic tissue is another option for the management. Wide local excision and debridement of all devitalized oral, nasal, sinus and orbital tissue with total exenteration of the eye ball may be performed for the benefit of the patient. However, this very extensive surgical procedure may not be practicable all the time. At the same time, orbital exenteration could sometimes be life-saving in patients with orbital zygomycosis.[79]

Influence of fungal species on clinical presentation, therapeutic management and outcome of infection

Of the all the etiological agents enumerated earlier, *Rhizopus, Mucor* and *Aspergillus* species are exclusively angio-invasive. Once sinus is involved by the fungus,[80] orbit becomes an easy access for these invasive microbes. The mode of orbital invasion has already been discussed in the section relating to pathophysiology. The clinical outcome of orbital cellulitis is invariably fatal unless appropriate and timely therapeutic measures are undertaken. This is especially so if there is intra-cranial spread, which is not unusual in an immuno-compromised patient. More importantly, *Mucor* and *Rhizopus* can give rise to a fulminant and acutely fatal disease when the patient is ketoacidotic. In such cases, the prognosis is very poor.

Although intravenous AB is the best, problem sometimes arises in managing the cases because of diagnostic dilemma. The cases with post-septal cellulitis are often confused clinically with orbital pseudotumors (idiopathic orbital inflammatory disease) and these individuals may unnecessarily be kept on steroids. Similarly an encysted orbital abscess may not always be of fungal origin, or one may find a rhabdomyosarcoma, being clinically

confused with orbital infection, in which case management strategies may be totally different.

In such clinical situations, diagnosis of orbital cellulitis can always be confirmed by ultrasonography and proper microbiological investigations. Even if mycological diagnosis confirms fungal infection of the orbit, still the managing the case sometimes is difficult. This is owing to the fact that fungal isolates from deep seated infections like this often show higher MICs for AB. Thus the patient might have to be put on a high dose regime i.e. 5mg/kg/day of the drug, with a constant watch on blood urea and creatinine levels. In case of blood chemistry abnormality, the drug should be replaced with oral pasaconazole.[81]

DACRYOCYSTITIS

Dacryocystitis is the infection of the lacrimal sac causing obstruction of nasolacrimal duct (NLD). As a result, the normal flow of tears through the sac is obstructed.

Pathogenesis

Stagnation of tears due to obstruction and resultant accumulation of debris in the lacrimal sac, act as the potential nidus for the organisms to propagate within the sac causing inflammation, hyperemia, edema and hypertropy of the mucosal epithelium. Accumulation of mucoid and mucopurulent exudate cause the sac to dilate, ultimately leading to a pyocele.[82] Thus, dacryocystitis consists of two components i.e. stasis and infection and form a vicious cycle. Fungi usually do not cause acute dacryocystitis. Chronic dacryocystitis is usually due to a single site of partial or complete obstruction within the lacrimal sac or within the nasolacrimal duct. Infection can rarely spread for the neighboring anatomical sites.

Etiological agents

Scattered documentations on the fungal cause of dacryocystitis show varying results. Fungi alone were found to account for only 5% of cases of acquired dacryocystitis and for almost 15% of cases of congenital dacryocystitis.[83] A study conducted at our centre revealed as many as 30.2% of the eyes in congenital dacryocystitis, *Candida albicans* and *Aspergilli* being the most frequent[68] (Table2). However, a recent study conducted at our centre showed fungi in 5.4% of 112 samples from congenital, and in 4.5% of 110 materials in acquired dacryocystitis cases[84] (Table 3 and 4).

Clinical features

Invariably, majority of patients present with epiphora and discharge. Discharge may be mucoid to mucopurulent or purulent. Other features which may be noted are those of conjunctivitis, mucocele or abscess and lacrimal fistula. On applying pressure on the lacrimal sac region, there is regurgitation of purulent material through the lower punctum. Whitish yellow to brown concretions (dacryoliths) may be seen at the lacrymal puntum.[85] Sometimes, the disease may run a mild and chronic course leading to a complete cicatricial obliteration of the lacrimal passage. The development of an encysted mucocele and even the formation of a fistula on the face are not uncommon.[85]

Laboratory diagnosis

The purulent material from the lacrimal sac is the samples for microbial study. If regurgitated material is inadequate, one can opt for draining the contents of the sac with a sterile syringe and needle. If lacrimal sac is removed by surgery, then it is bisected for histopathology and culture. Like a biopsy material, it is then ground, suspended in sterile buffered saline and used for culture.[81] Isolation identification procedure is the same as described above for fungal keratitis**.**

Treatment of dacryocystitis

Mycotic dacryocystitis responds well to topical administration of 5% Natamycin. Sometimes, alongwith the topical application, local syringing of the sac with either Amphotericin-B (1.5 to 8mg/ml) or Nystatin (100,000 units/ml) solutions may be quite helpful.[83] The recommended surgical procedures adopted are probing and syringing of the obstruction and dacryocysto-rhino-stomy(DCR).[84,86]

Influence of fungal species

In *Candida* infections, the concretions described above are yellowish white in color and of rubbery consistency,[87] whereas, those due to *Aspergillus niger* are brown to black. Recently, it was documented that higher rate of culture positivity (both fungal as well as bacterial) was seen, when the discharge was mucoid to muco-purulent.[71] Mycotic dacryocystitis invariably responds satisfactorily to topical antifungals. However, if medical management fails, surgery is advocated. In spite of the above mentioned effective antifungal regime, the clinical outcome of fungal dacryocystitis, especially those due to *Aspergilli* or *Candida* is not quite encouraging. About 40% of the cases managed by medical treatment alone do recur, whereas around 80% of those who undergo DCR along with medical treatment get cured.[83,84]

ENDOPHTHALMITIS

Endophthalmitis is an inflammatory reaction of intra-ocular fluid or tissues. It can be both infectious and non-infectious. Infectious (post-operative, post-traumatic or endogenous) endophthalmitis is one of the most serious and vision threatening complications of ophthalmic surgery.[88]

Etiological agents

There are varying reports on fungal etiology of endophthalmitis. In post-operative cases, *Aspergillus*, *Fusarium, Alternaria* are reported to be the commonest agents[88-90] whereas in endogenous cases, *Aspergillus* and *Candida* have been incriminated.[88,91] *Aspergillus, Alternaria, Bipolaris, Acremonium, Fusarium* are mostly

accounted for post-traumatic cases.[88,92] *Histoplasma capsulatum* var. *capsulatum* and *Coccidioides imitis* are also reported in metastatic endophthalmitis and should be considered in endemic places and in immunocompromised patients.[93,94] Overall positivity of fungal isolation in infectious endophthalmitis is reported to be vary between 11 to 16%.[86-88]

Relevant risk factors

The endogenous endophthalmitis have a variety of associated pre-morbid conditions that may pre-dispose them to infetion.[88] Most of the fungal endophthalmitis is associated with immunosuppression. These include diabetes mellitus, leukaemia, lymphoma, alcoholism, AIDS, prematurity, IV drug abuse, parenteral hypheralimentation and long term antibiotic therapy. Amongst the various traumatic factors which precipitate post-traumatic endophthalmitis include lens disruption, intra-ocular foreign body, plant and soil related injury, injury in rural setting and penetration with an obviously contaminated device.[88]

Endophthalmitis following surgery, however, is influenced by various factors, including pre-operative conditions, like canaculitis, dacryocystitis and contact lens use; intra-operative, like profound vitreus loss, prolonged surgery and inadequate eye lid/conjunctival disinfection; and post-operative conditions such as wound leak or dehescences, inadequately buried sutures following blebs and silicon lenses.[91]

Endophthalmitis resulting from contact lens use[68] needs special mention. There seems to be a higher correlation between fungal contamination of contact lens solution and development of fungal keratitis and endophthalmitis, donor corneo scleral rim and post-operative infection. Although the overall incidence of fungal infection following penetrating keratoplasty is low (0.16%)[7] there seems to be a higher correlation between fungal contamination of donor corneocleral rim and post-operative infection, mostly due to *C. albicans* and other *Candida* species[7] as well as due to filamentous fungi such as *Exophiala dirmatitidis*.[95]

Thus in most eye banks, cold storage at 4°c is practised.[7] Storage medium in most of the cases is tissue culture medium with antibiotics like gentamicin and streptomycin or AB, along with Penicillin and Streptomycin.[96,97] During storage, a screening for any microbiological contamination of donor corneal medium is always employed. If contamination is detected, those are discarded. Viable non-contaminated donor corneas are further incubated for 24 hrs and are used for surgery if no turbidity is noted. It is debatable if a prolonged period of warming of the donor button in storage medium could reduce the incidence of fungal contamination**.** The method of disinfection of donor globes may also affect the rate of contamination of donor corneas. It is advocated that use of 1% solution of povidone-iodine to decontaminate the globe resulting in significant reduction in microbial growth, especially that due to *Candida* species.[98]

Clinical features

Fungal endophthalmitis symptoms are similar to those seen in bacterial one. These include, blurring of vision, pain, photophobia and red eye.[99,100] Important external signs of inflammation include ciliary congestion, chemosis, lid oedema, raised intra-ocular pressure, restrictionof external ocular mobility and proptosis. Intraocular signs include diminshed visual acuity, altered pupillary defect, increased pain and redness, hypopyon, corneal oedma, corneal infiltrate, retinitis, severe vitreous inflammation with persistent iritis alongwith visible puff balls and strands**.** However, there are certain features which are unique to fungal infection. In *Candida* endophthalmitis, for example, there is a creamy, white, well circumscribed lesion involving the choroid and retina.[91] The lesions may be multiple, are most often located in the posterior pole and have associated retinal haemorrhage and perivascular sheathing. The vitreous may contain yellow-white opacities in the form of 'string of pearls' or fluff balls.[88,91] It is also true that, severe vitreous inflammation with persistent iritis alongwith whitish fluff balls and strands are quite characteristic of mycelial fungal endophthalmtis.[88] Other findings include hypopyon and optic nerve edema.

Laboratory diagnosis

Due to being a vision threatening condition, prompt and rapid laboratory diagnosis is very important in endophthalmitis. If there is a delay in the management and in the administration of appropriate antimicrobial agents, one may have to go for making a decision for evisceration of the eye ball. Therefore, when the patient presents with signs and symptoms suggestive of infectious endophthalmitis, the best approach is to obtain intra-ocular sample for microbiological investigation. Secondly, any post-operative inflammation, which is more severe than is normally expected after intra-ocular surgery and is unresponsive to a course of intensive topical corticosteroids is always suspicious and requires immediate culture of intra-ocular fluid. Thirdly, presence of hypopyon after intra-ocular surgery is a strong indication for vitreous biopsy/culture. In addition, breaded anterior segment or vitreous opacities with strands are quite suggestive. In all the above clinical situations, samples should be collected without delay so that prompt laboratory diagnosis can be made. The various clinical samples which are of help include (a) anterior chamber aspirate (b) vitreous tap (c) vitrectomy/ vitreous biopsy specimen.

Collection and transport of specimens: Anterior chamber aspirate, though not as helpful as vitreous sample, is sometimes useful in making a diagnosis. About 0.2 to 0.3ml of aqueous is collected as described

above in the section for keratitis. Vitreous fluid is collected either by vitreous needle tap or by vitreous biopsy. Vitreous needle tap is best collected by a sterile tuberculin syringe and a 22 gauge needle by approaching the anterior portion of vitreous cavity through pars plana region. About 0.1 to 0.3ml of fluid is collected by manual aspiration. At times, due to severe vitreous inflammation, it is not possible to collect vitreous aspirate by simple needle tap. Hence, vitreous biopsy is the only alternative. In this procedure, vitreous is cut with a vitrectomy cutting/aspirating probe, which is attached to a tuberculin syringe and needle. Vitreous cavity is reached through pars plana approach. Nearly 0.2 to 0.3ml material is obtained by manual aspiration into the syringe during the activation of the cutting mechanism. The specimen is sent to the laboratory, preferably undiluted to increase the yield.

As mentioned earlier, the aqueous and/or vitreous specimens are usually sent to the laboratory in the same syringes used for collection. If the laboratory is not located nearby the culture media need be inoculated in the operating room itself. If bedside inoculation is not possible due to some reason and delay in transport is apprehended, then the sample should not be refrigerated. It is preferable to preserve the sample in a 25°C in BOD incubator, till it is processed. Both aqueous and vitreous cultures are recommended in endophthalmitis.[101,102] However, the sensitivity of the culture is increased with the vitreous rather than with the aqueous alone.[102] Cultures of external ocular surfaces are not of value except in the presence of an open wound or a leaking bleb. The advent of therapeutic vitrectomy has provided an alternative modality of obtaining vitreous in endophthalmitis.

If a two or three port vitrectomy is done for collecting vitreous biopsy, then vitrectomy cassette fluid is the ideal sample for culture. The sensitivity of culture of vitretomy casstte fluid, passed through a 0.2u Millipore filter, is reportedly higher than vitreous biopsy culture obtained by a needle and syringe.[102]

***Processing and identification of fungi*:** The procedure of microscopic examination and culture is the same as used routinely for any other ocular specimen described above. Smear examination though provide a rapid diagnosis[103] is very less sensitive. The commonly used Gram's and Giemsa's staining techniques have 60% and 41% sensitivities respectively.[104]

Apart from conventional culture, the membrane filter system is advocated for better yield of the micro-organism.[103] The vitrectomy specimen is first processed by passing through a 0.2u membrane filter. The filter is then removed aseptically and cut into segments for direct inoculation onto the culture media. Processing of both vitreous biopsy and vitrectomy cassette fluid by this technique provides greater sensitivity.[104]

***Limitations of fungal culture*:** There are certain limitations to culture in establishing an etiological diagnosis of fungal endophthalmitis. First of all, the sample size is very small, and organisms being in a fluid sample are diluted and small in number. So, a little delay in processing may result in loss of viability of the organisms. Secondly, fungi are ubiquitous and chances of laboratory contamination can be a possibility. So, one should always inoculate more than one fungal culture media for the same sample. Unlike in case of fungal keratitis, repeat sampling is not possible in this case for confirmation of the aetiological agent. Thirdly, review of the major reports in the literature shows that only 64% of vitreous specimens obtained from eyes with clinical diagnosis of endophthalmitis are culture positive.[103] Fourthly prior use of antibiotics may yield negative results in culture and lastly it should always be remembered that fungi are slow growing organisms and so there is always a time lag between processing of the sample and getting a positive culture.

***Molecular methods*:** Considering the limitations of culture methods, molecular methods such as PCR in the rapid diagnosis of fungal endophthalmitis seems quite promising. Most of the PCR techniques use the multi copy gene targets such as the fungal ribosomal DNA gene cluster.[105] The ribosomal DNA (rDNA) gene is a tandem array of at least 50-100 copies in the haploid genome of all fungi. It comprises the small subunit rDNA (18S) gene, the 5.8 S gene and the large subunit rDNA (28S) gene.[35] Separating the 18S and 5.8S is ITS1 region and 5.8S and 28S is ITS2 region, which are called internal transcribed spacer regions. Between each of these set of transcripts is the intergenic spacer region (IGS). Any component of this gene cluster can be selected as a target for PCR. Whereas rDNA genes (coding regions) are highly conserved, the ITS regions are moderately variable and the IGS region is highly variable between different fungi.[106] So this allows the designing of universal primers based on the conserved regions, which will amplify a certain region of the rDNA gene cluster from a large number of fungal species, as well as species specific primers/probes based upon the variable regions, that can be used to identify the species. At least 16 *Candida* and 5 *Aspergillus* species specific probes have been designed based upon this principle.[26,107]

Anand *et al*[108] recently studied 27 intra-ocular specimens from 22 cases of suspected fungal endophthalmitis, by conventional microscopy and culture as well as by PCR assay, for the detection of fungi. None of the controls (non-infective intra-ocular disorders) were positive. PCR detected fungi in more number of samples, which were negative by the conventional method. Average time required for culture was 10 days, whereas PCR needed only 24 hours. In another study, PCR was found to be more sensitive and a rapid diagnostic tool compared with the conventional mycologic methods in the diagnosis

of fungal endophthalmitis.[109] Hidalgo *et al,*[110] in yet another study of *Candida* endophthalmitis noted PCR positivity in all the four patients of suspected endophthalmitis, by using species-specific PCR in the vitreous samples, while the culture of vitreous was negative in two specimens.

Thus, it appears that PCR is a highly sensitive and rapid method to diagnose fungal endophthalmitis. It is a very useful laboratory tool, especially in the tropical countries, where incidence of fungal eye infections is quite high. The much more rapidity of PCR as compared to culture will certainly help the Ophthalmologist in planning the effective and quick therapeutic measures. Its paramount effectiveness in the rapidity and accuracy in diagnosis will ultimately have a major impact on improvement in the prognosis of patients with fungal endophthalmitis. This is especially true for post-operative endophthalmitis following cataract surgery, which is the commonst form of fungal endophthalmitis, where early diagnosis is very important for effective management of patients in order to avoid severe and vision threatening ocular morbidity. According to a recently conducted study in India,[111] it was observed that PCR for detection of fungal DNA was found to be a rapid and a more sensitive method compared to conventional culture method in the early diagnosis of post-operative endophthalmitis. In addition, further areas of interest include identification of fugnal species using species specific primers.[106,112,113] for the detection of *C. albicans*, *A. fumigatus*, *F. solani* and *Al. infectoria*[114] in intraocular samples as well as in corneal scrapings**.** This would ultimately have a major impact on improvement in the prognosis of the subject not only with fungal endophthalmitis but with other ocular fungal infections as well.

Influence of fungal species on clinical presentation, therapeutic management and outcome of infection

Endophthalmitis due to *Candida* species is well documented, both as a consequence of fungemia and as a result of dissemination from endogenous source in an immunocompromised indivisual. Intra-ocular signs and symptoms such as diminished visual acuity, severe vitreous inflammation with persistent iritis, whitish puff balls and strands are more commonly seen in endophthalmitis due to *Candia* and *Aspergilli*. In addition, infection due to *C. albicans* may result in choroidal neovascularisation, which is a potential cause of late visual loss in patients who have had sepsis and endogenous chorioretinitis due this organism.

The therapeutic implications of the above clinical entity are alarming. Species of *Candida* other than *C. albicans* are reportedly showing in vitro resistance to Fluconazole. In addition, *C. tropicalis* is intrinsically resistant to many azole compounds. Thus, newer azoles like voriconazole and posaconazole are worth trying.[52]

CONCLUSION

Ocular fungal infections are increasingly gaining importance in clinical practice due to modern therapeutic management facilities and increasing number of immunocompromised patients. Thus our research needs to focus entirely on improvement in diagnostic techniques, development of new antifungal agents and standardization of their sensitivity testing and a better understanding of the pathogenesis of the conditions.

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