OCULAR FUNGAL INFECTIONS

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DEDICATION FROM AYSE KALKANCI

I dedicate this book to my family; my beautiful Defne and my sweetheart Bora, my loving parents Leman and Cengiz Seçkin, my dearest sisters Hande and Dilek, their wonderfull husbands Barış and Rasim, and my smart nephew Zeynep.

DEDICATION FROM SENGUL OZDEK

To my invaluable family.

DEDICATION FROM MEHMET CUNEYT OZMEN

In memory of my father…

For the three beautiful women of my life; my mother, my wife, and my daughter.

FOREWORD

Infectious diseases of the eye have been recognized as an important cause of blindness. As a relatively uncommon cause, fungi have been isolated from a variety of ocular infections including keratitis, scleritis, canaliculitis, endophthalmitis and orbital cellulitis. Fungi are recognized as opportunistic pathogens. Ocular fungal infections (ophthalmic mycoses) are important causes of morbidity, blindness and even mortality especially in tropical countries.

This book will focus on laboratory diagnosis and experimental models of ophthalmic mycoses as well as clinical features of fungal keratitis, endogenous and exogenous endophthalmitis. An outline of ocular anatomy will be given before detailing fungal infections.

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1. OCULAR ANATOMY (Figure 1)

Eye can be divided into 3 segments for the purpose of education as:

1. Eyelids and lacrimal system,
2. Orbits and adjacent soft tissues,
3. Eyeball: anterior and posterior segments

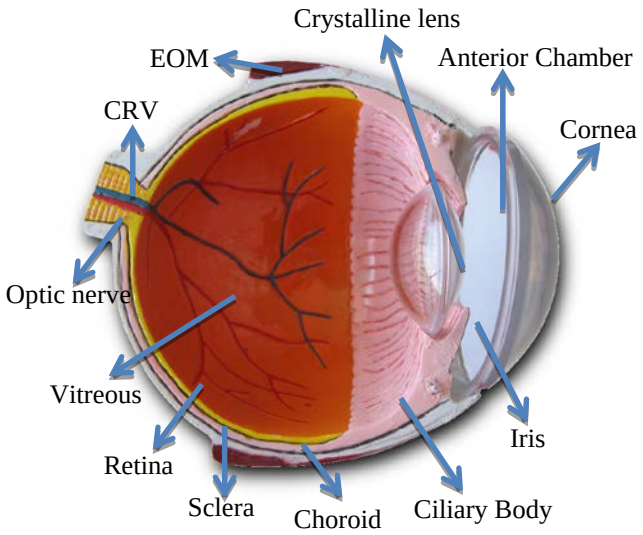

FIGURE 1. Anatomy of the eyeball. (EOM: Extraocular muscle, CRV: Central retinal vessels)

Eyelids are protective barriers for the globe. They protect the globe from dangers of the outside world. There is an orbital septum in each eyelid which acts as a barrier for the prevention of spread of infections through the orbital soft tissues. Conjunctiva, the innermost lamella of the eyelid and outermost structure of the globe, is another barrier for microorganisms on the anterior surface of the globe from freely entering into globe or orbital soft tissue along its surface. Lacrimal system is composed of a secretory part; lacrimal gland, accessory lacrimal glands in conjunctiva, and an excretory part; starting from puncta, canaliculi, lacrimal sac, nasolacrimal canal ending in the inferior meatus of the nose. Tears protect the globe from infections by rinsing the surface of the eye.[1]

Orbits are cone shaped bony cavities which involve the globes, extraocular muscles, fat and other soft tissues. They consist of seven bones forming a safe room for the

globes. The periosteum of the orbita fuses anteriorly with the orbital septum and posteriorly with the dura mater. Abscesses usually localize in the subperiosteal space. The roof, medial wall, and floor of the orbit are neighbours of paranasal sinuses, (the maxillary, frontal, ethmoid, and sphenoid sinuses). The paranasal sinuses may be the source of an orbital infection because of this close anatomical relationship. Medial orbital wall is the thinnest of the orbital walls and is the weakest point for the orbits. Infections of the ethmoid sinus in children commonly extend through the intact lamina papyracea (medial wall) causing preseptal and orbital cellulitis. The lateral wall of the sphenoid is also the medial wall of the optic canal. Therefore, infections of the sphenoid sinus may involve the optic nerve, resulting in visual loss or visual field abnormalities. Direct communication between the orbit and adjacent structures, through the apertures like the superior and inferior orbital fissures, nasolacrimal duct, and the optic canal may serve as a direct passage for an infectious process between the orbit and surrounding structures.[1]

Eyeball is composed of 3 layers: outermost is the fibrous layer consisting of cornea and sclera, middle layer is uvea consisting of iris (anterior part), ciliary body (middle) and choroid (posterior), and the innermost layer is retina. Crystalline lens and iris divides the eyeball into chambers like anterior and posterior chambers which are full of aqueous humor secreted by nonpigmented epithelium of the ciliary body, and vitreous space which is full of gel like vitreous. [1]

Defense mechanisms of the eye start from eyelids, eyelashes, tear film, cornea and conjunctiva with blink reflex and by providing mechanical barrier. In addition to mechanical washing of the ocular surface, tear film contains several immunologically active substances necessary for ocular defense. The mucin contained in tears prevents adhesion of *Candida* species to contact lenses, likely by entrapping the microorganisms.[2] Fungal infections of the eye will be discussed according to the anatomical part of the eye involved in the disease.

2. ETIOLOGICAL AGENTS AND EPIDEMIOLOGY

The incidence of ocular fungal infections has increased substantially over the past decades because of the increased number of patients with acquired immunosuppression secondary to extended use of immunosuppressive agents, long term broad spectrum antibiotics and AIDS.[3-7] The pathogenesis of eye infections is linked to the epidemiology of disease. The term of endogenous endophthalmitis indicates to blood borne spread of microorganisms into the eye. Mainly, neutropenic immunosuppressive patients undergo blood borne infections and fungemia. *Candida* species are the most common cause of endogenous endophthalmitis which usually develop in immunocompromised patients having chronic underlying systemic disease, an associated septicemia for which broad spectrum systemic antibiotic therapy is being administered, intravenous hyperalimentation with chronic indwelling catheters or an organ transplantation that requires immunosuppression.[8-10] Intravenous drug abusers, patients with diabetes and AIDS are also at high risk for endogenous fungal endophthalmitis (FE). Abdominal surgery is another risk factor for candidemia and hence for endophthalmitis. Common end organ target of fungemia is eye in many cases. But the reason of this tropism is unknown.[11-13]

Aspergillus species are the second most common cause of endogenous fungal endophthalmitis. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *A. glaucus*, *A. nidulans* have been reported to cause endophthalmitis. Neutropenic patients or patients receiving corticosteroids, intravenous drug addicts, solid organ transplant recipients are at particular risk for endogenous endophthalmitis with *Aspergillus* species.[12,14]

There are several reported cases representing other emerging pathogens such as *Fusarium, Penicillium, Pseudallescheria, Cryptococcus* species, dimorphic fungi *Histoplasma capsulatum, Blastomyces dermatitidis, Sporothrix schenckii, Coccidioides immitis* caused endogenous endophthalmitis.[12]

Exogenous fungal endophthalmitis occurs by inoculation of pathogens into the eye from, trauma or intraocular surgery and usually follows keratitis. Patients with exogenous endophthalmitis are rarely immunocompromised. Therefore any of the

saprophytic fungi found in natural habitats, may cause exogenous infection of the eye. The mycotic causes of exogenous endophthalmitis are mainly *Candida* species especially in postsurgical group[5,14], whereas *Fusarium* species were found only in the posttraumatic and postkeratitis groups.[15,16] An epidemic of postsurgical endophthalmitis with *Candida parapsilosis* has been reported representing 15 patients had ocular surgery over a 3-month period of time.[17] At the time of surgery all eyes were learnt to be irrigated with a solution from the same lot that was contaminated with *C. parapsilosis*.

Paecilomyces, *Aspergillus*, *Acremonium*, *Exophiala*, *Pseudallescheria*, *Syctalidium*, *Sporothrix*, *Penicillium* species were also reported as the etiological agents of exogenous endophthalmitis cases.[18] *Fusarium* species were the most prevalent (30%) organisms, followed by *Aspergillus* species (13.3%), *Acremonium* species (8.3%) and *Paecilomyces* species (8.3%). Other moulds were the causative agents only in 13.3% of the cases.[18,19] *Candida* species were more prevalent especially in postsurgical group, whereas *Fusarium* species were found only in the posttraumatic and postkeratitis groups.[16,20]

Fungal pathogens in posttraumatic endophthalmitis are numerous and similar to those causing fungal keratitis. Reports include *Exophiala jeanselmei, P. boydii, A. niger, Scytalidium dimidiatum, Helminthosporium* spp., *S. schenckii,* and *Penicillium chrysogenum.*[18,19]

Fungal keratitis or keratomycosis is the third clinical presentation of ocular fungal infections. Wearing of hard and soft extended-wear contact lenses is associated with bacterial infections usually caused by *Pseudomonas aeruginosa*.[14] Fungal keratitis usually occurs after trauma with fungus-contaminated plant material in agricultural workers. Majority of cases are due to soil saprophyte filamentous fungi belonging to nearly 56 genera have been reported from the cases of corneal infections. These are *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus, Aureobasidium pullulans, Alternaria alternata, Cladosporium oxysporum, Cylindrocorpon tonkinensis, Curvularia lunata, Curvularia geniculata, Curvularia pallescens, Curvularia senegalensis, Curvularia verruculosa, Cladorrhinum* spp., *Drechslera*

spp., *Drechslera rostrata, Drechslera spicifera, Lasidiplodia theobromae, Lichtemia* spp. (Formerly *Absidia,) Phialophora verrucosa, Phoma oculohominis, Pleospora infectoria, Botryodiplodia* spp., *Tetraploa* spp., *Rhizoctonia* spp., *Rhizopus* spp., *Macrophoma* spp., *Trichosporon* spp., *Ustilago* spp., *Scopulariopsis* spp., *Pseudallescheria (Syn. Allescheria) boydii, Sporothrix schenckii, Verticillium* spp., *Acremonium* spp., *Fusidium* spp., *Sterigmatocystis nigra, Paecilomyces lilacinus, Periconia keratitidis, Neurospora* spp., *Volutella* spp., *Glenospora* spp., *Penicillium* spp., *Penicillium citrinum, Penicillium spinulosum, Graphium* spp., *Fusarium solani, Fusarium nivale, Fusarium oxysporum, Candida albicans, Candida guilliermondii, Candida viswanathii, Candida krusei, Rhodotorula* spp., *Colletotrichum state of Glomerella cingulata, Acrophialophora fusispora, Phaeotrichoconis crotalariae, Helminthosporium, Neosartorya fischeri var. fischeri, Arthrobotrys oligospora, Trichophyton mentagrophytes, Epidermophyton floccosum, Scedosporium apiospermum*.[22,23] First report of fungal keratitis caused by *Carpoligna pleurothecii* was published recently.[24] Fungal infections of the cornea are relatively infrequent in the developed world, but constitute a larger proportion of keratitis in many parts of the developing world especially tropical countries.[20,23,25-28] *Candida* and *Cryptococcus* may cause fungal keratitis in patients with chronic dry eye syndrome, chronic ulceration, erythema multiforme and human immunodeficiency virus infection. Fungal keratitis may constitute 6 to 53% of all cases of ulcerative keratitis, depending on the country of origin of the study.[27-29] Fungal keratitis is a major blinding eye disease in Asia.[6,7,27] In temperate climates, such as Britain and the northern United States, the incidence of fungal keratitis remains very low. Corneal trauma contaminated with plant material is the most common scenario for fungal keratitis. Filamentous fungi, such as *Fusarium solani* and *Aspergillus flavus*, may constitute up to one-third of all cases of traumatic infectious keratitis.[21-30] In the northern parts, however, *Candida* infections predominate and corneal disease and local/systemic immunosuppression are associated with these infections.[23]

Since 1980s, contact lens wear has been increasingly recognized as a risk factor for *Fusarium* keratitis. There are a number of reported outbreaks of *Fusarium* keratitis

among contact lens wearers in Singapore, Hong Kong, USA, Puerto Rico, Caribbean region.[29]

Invasive *Aspergillus* and Mucoromycotina (*Zygomycetes*) infections have a marked predilection for the orbit and surrounding tissues, including the paranasal sinuses.[30,31] Many different presentations of eye disease by *Aspergillus* occur even in the healthy host, being more invasive in immunocompromised host. Invasive zygomycosis, "rhino-orbito-cerebral (ROC) Mucoromycotina infections" is a devastating complication of diabetic ketoacidosis and the use of immunosuppressive drugs following organ transplant.[31]

Disease	Fungus	Risk factors or comments
Endogenous endophthalmitis	*Candida* spp *Aspergillus* spp Dimorphic agents	Neutropenia Broad spectrum antibiotics Central venous catheters Intravenous drug users Abdominal surgery
Exogenous endophthalmitis	*Candida* spp *Paecilomyces* spp *Fusarium* spp	Postoperative infection Posttrauma
Keratitis	Filamentous septated fungi (*Fusarium* and *Aspergillus* spp) Filamentous non-septated fungi (*Mucor* and *Rhizopus* spp) *Candida* spp	Trauma Superinfection of cornea Prolonged corticosteroid use

Table 1: Most frequently isolated fungal agents causing ocular mycosis. (Adapted from Klotz SA et al)[3]

3. CLINICAL DIAGNOSIS AND TREATMENT

3. 1. FUNGAL ENDOPHTHALMITIS

Fungal endophthalmitis is an acute or chronic intraocular inflammation caused mostly by *Candida* and *Aspergillus* species. Fungi can cause both endogenous and exogenous endophthalmitis. The incidence of bloodborne fungal infections may have increased with the increased number of immunosuppressed patients as well as the use of newer more potent broad-spectrum antibiotics that reduce normal flora[8-14] Table 1 lists most frequently isolated fungal agents causing endophthalmitis and related risk factors.

3. 1. 1. Endogenous Fungal endophthalmitis

Infection in the eye is the result of metastatic spread of infection from a distant site, for example, infected heart valves or the urinary tract. Endogenous endophthalmitis has been associated with many systemic risk factors mentioned above. Ocular presentation of the most commonly encountered agent *Candida* includes a creamywhite, well circumscribed cottonlike lesion, resembling a “fluff ball,” involving the retina and choroid and extending into the vitreous cavity.The lesion usually is less than 1 mm in diameter, often localized in the posterior pole, and associated with overlying vitreous inflammatory cells (Figure 2a).[13] The infection is called as *Candida* chorioretinitis when the infection remains localized in the retina and choroid (Figure 2a), however, if it extends into the vitreous as an abscess or fluff ball with vitreous haze, it is then referred to as *Candida* endophthalmitis (Figure 2b).

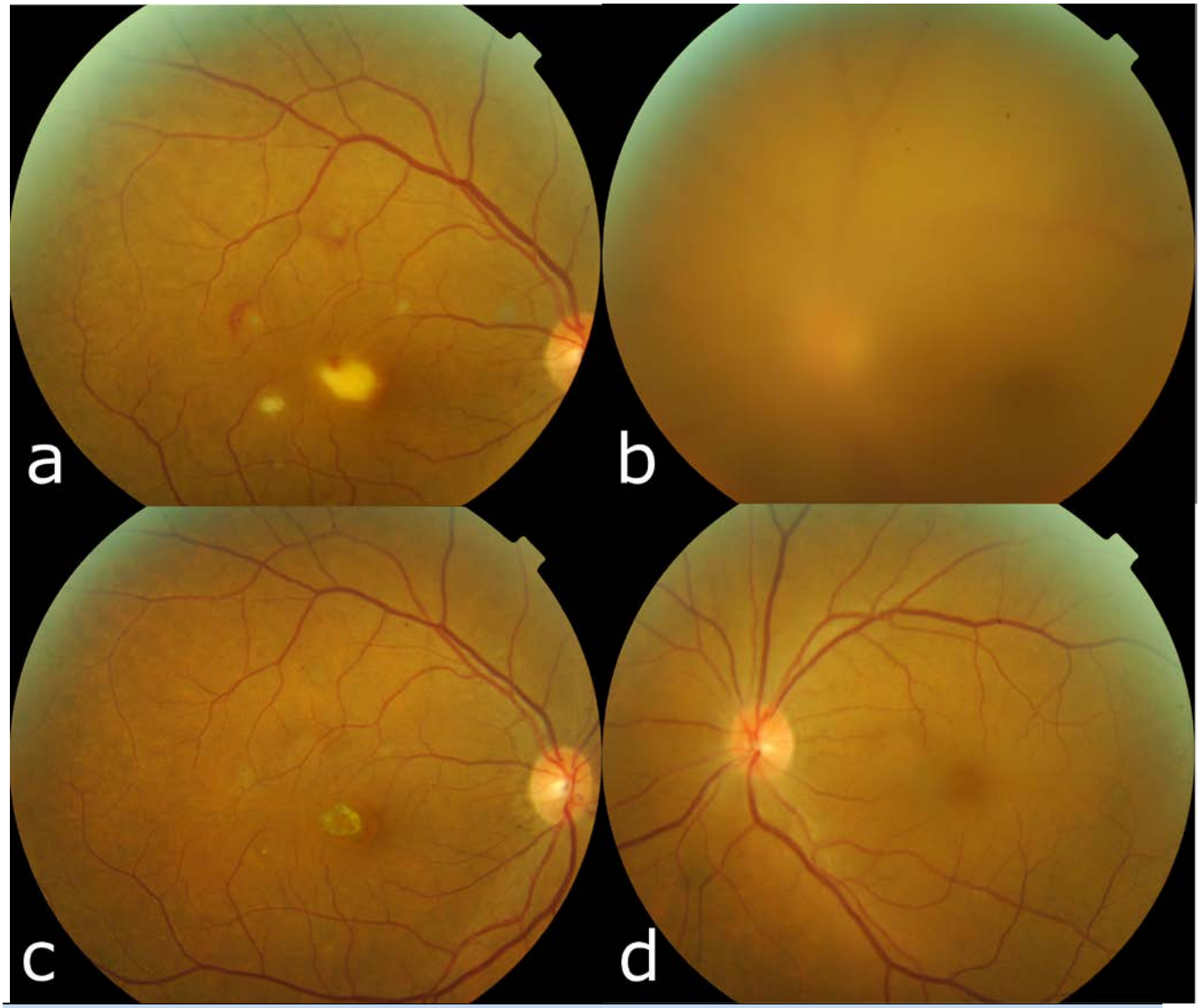

FIGURE 2. Endogenous *Candida* chorioretinitis foci in the right eye (a) and significant vitritis (endophthalmitis) in the left eye (b) of a case with pancreatic head tumor having chemotherapy[13]. After intravenous fluconazole treatment and cessation of chemotherapy lesions in the right eye regressed (c). In addition to intravenous fluconazole and cessation of chemotherapy, pars plana vitrectomy and intravitreal amphotericin B treatment resulted in recovery of the left eye (d).

More than one half of patients will have vitreous involvement. Vascular sheathing of the retinal vessels may be present, and an associated iridocyclitis is common. One to two thirds of patients have bilateral involvement of the fellow eye, and one half of patients have multiple lesions when first examined.[32-36] Multiple yellow-white vitreous abscesses are classically referred to as a “string of pearls”. Figure 3 and 4 shows typical pictures of these pearls in our culture proven *Candida* endophthalmitis cases. *Candida* chorioretinitis is the most common fungal infection of the retina.

Animal studies have found that *Candida* may have a greater propensity for the eye than other species of fungi do.[37]

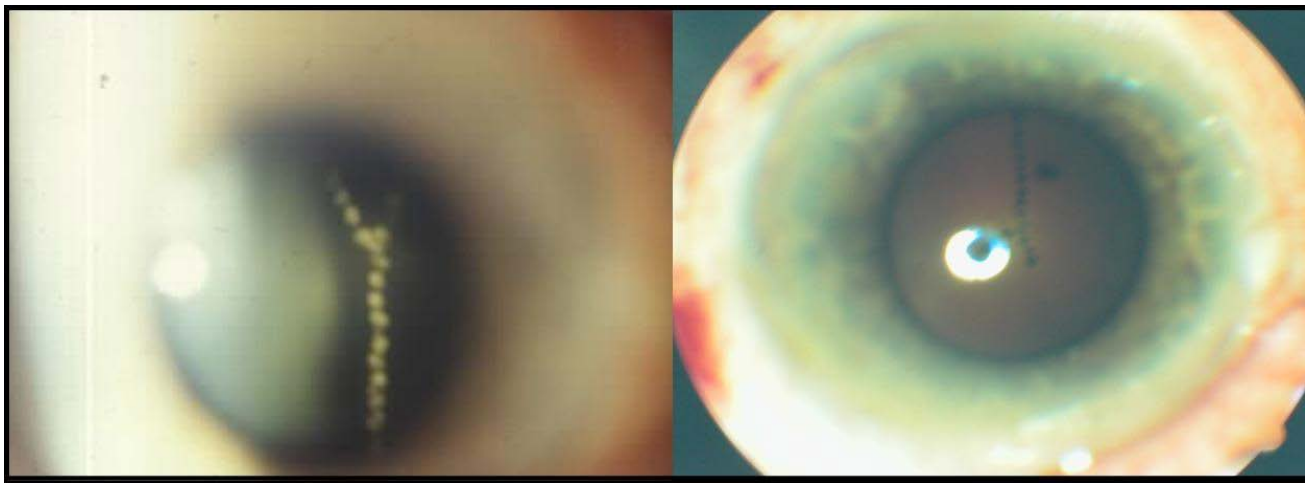

FIGURE 3. Pearls in the vitreous cavity seen through pupilla in a bilateral endogenous *Candida albicans* endophthalmitis case.

The most common symptom at presentation of endogenous FE is decreased vision. Red eye may be absent in more than half of the cases.[8] *Candida* endophthalmitis can masquerade as uveitis and have a gradual onset with a relatively indolent course. It was found to be associated with an incorrect initial diagnosis of uveitis which may reach up to 50%.[32,33] This underscores the need for the ophthalmologist to maintain a high suspicion of endogenous endophthalmitis for patients with intraocular inflammation and a recent history of hospitalization, significant medical comorbidities, or a history of *Candida* infection.[35-38]

Diagnosis of endogenous FE is often difficult depending onculture results and therefore limited to clinical findings. Cultured fungus from the vitreous confirms the diagnosis but is rare because the organism often is confined to the retina and only inflammatory cells are found in the vitreous itself.[38] Blood and urine cultures may also confirm the diagnosis when the infection is known to be endogenous. However, because of the prolonged culture time, slow growing or fastidious fungal organisms are often undetected. Binder et al. showed that, aside from blood and eye specimen cultures, half of patients showed an additional systemic infection, most frequently a

urinary tract infection.[33]

Aspergillus endophthalmitis may be encountered especially in patients with neutropenia, taking pharmacologic doses of corticosteroids (often for chronic lung disease) and intravenous drug addicts.[12,30] Patients with endogenous FE caused by *Aspergillus* generally had worse visual outcomes compared with those caused by *Candida* species.[32,33] In cancer cases, on the other hand, spectrum of fungal agents causing FE may be completely different. Lamaris et al. has reported a review of 23 FE cases in a tertiary care cancer center, 65% of which being induced by molds like *Fusarium*.[8]

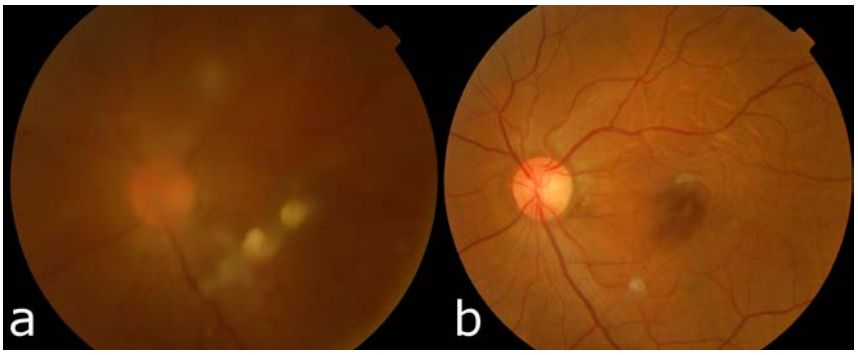

FIGURE 4. Pearls in the vitreous cavity seen through pupilla (a) in a bilateral endogenous *Candida albicans* endophthalmitis case. With vitrectomy and intravitreal amphotericin B injection visual acuity improved from counting fingers to 20/100 (b).

The optimal treatment of endogenous FE has yet to be established. Treatment of the main focus of infection such as an infected catheter should be the first line of treatment, however most patients require treatment with systemic antifungal treatment (Figure 2a,c). In severe cases not responding to systemic treatment, intravitreal therapy and pars plana vitrectomy should be considered (Figure 2b,d). Early treatment during chorioretinitis stage is more likely to result in better visual outcome. The choroid and retina are highly vascularized tissues which suggest that systemic pharmacotherapy may be sufficient to treat infections confined to these structures but severe involvement of vitreous may require intravitreal treatment.[35] Intravenous administration of amphotericin B has been the drug of choice

for the treatment of endogenous FE previously. However, because of the systemic toxicity and side effects of amphotericin B (especially nephrotoxicity), oral triazoles has become an alternative treatment for endogenous FE. Fluconazole and voriconazole are tolerated well, have a long half-life, have good intraocular and vitreous penetration, and has no reported ocular toxicity.[9,34,35,39,40] If the therapeutic response is not satisfactory with fluconazole, intravenous (and intravitreal) amphotericin B can be the treatment of choice. Administration of voriconazole has been associated with favorable outcomes.[40] Intravenous antifungal treatment is the mainstay therapy and should be continued for a long period of time with careful ophthalmic and systemic evaluation of the patient. Lastly, pars plana vitrectomy is an effective treatment option in eyes unresponsive to medical treatment. Vitrectomy has also a diagnostic value in indeterminate cases. Very recently combination of moxifloxacin to liposomal amphotericin B, has been reported to add to the antifungal activity in an experimental *C. albicans* endophthalmitis model.[41]

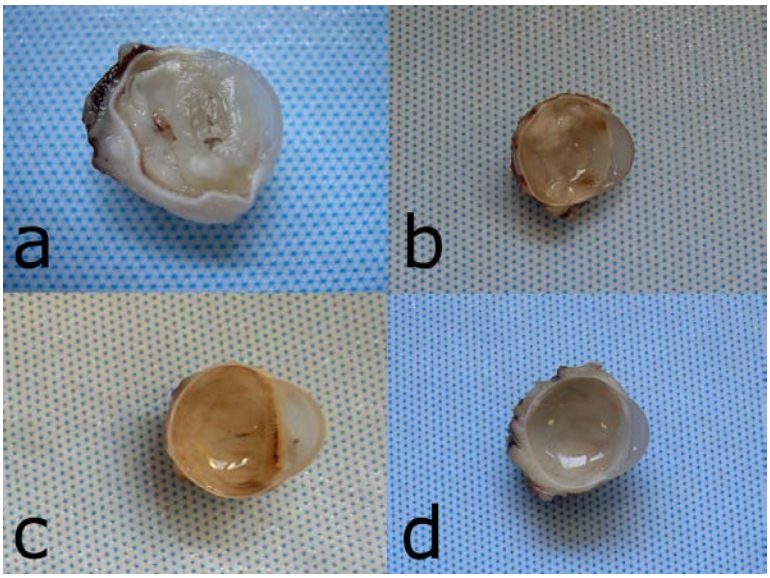

Figure 5. Section of rabbit eyes of a *Candida albicans* endophthalmitis model. A rabbit study conducted in Gazi University showed decrease of tissue damage when moxifloxacin is used in combination with amphotericin B.[41](Control group without treatment (a). Study group treated with moxifloxacin only (b). Study group treated with amphotericin B only (c). Study group treated with amphotericin B and moxifloxacin (d).)

The prognosis of endogenous FE remains unfavorable, as it is associated with poor visual acuity as well as high overall mortality rates.[8,42] Patients with systemic candidemia associated with a debilitating disease, may have a high mortality rate.

Mortality rate was reported to be as high as 77 % among patients with *Candida* endophthalmitis and known systemic candidemia, suggesting that ocular involvement is a good predictor of mortality for systemically ill patients.[42] Four-week mortality was reported as 57% in cases with FE associated with malignancy, being highest (73%) especially in those caused by moulds.[8] Most patients are seriously ill and hospitalized; however, any patient with intraocular inflammation and a history of recent hospitalization or systemic risk factors should raise suspicion of endogenous FE. Unfortunately, visual outcomes remain largely influenced by the causative organism, with *Aspergillus* having the worst prognosis.

Fungi	**Risk factors**
Candida spp	Diabetes mellitus, neutropenia, hyperalimentation, gastrointestinal surgery, prior antibacterial agents
Aspergillus spp	Transplant recipients, neutropenia
Fusarium spp	Neutropenia, intravenous drug abuse, AIDS
Cryptococcus neoformans	AIDS
Penicillium spp	Intravenous drug abuse, *Penicillium*-related endocarditis
Coccidioides immitis	Patients with dissemianted disease, may occur in otherwise healthy individuals.

Table 1:Most frequently isolated fungal agents causing endophthalmitis and related risk factors

3.1.2. Exogenous Fungal Endophthalmitis

As the name implies, exogenous endophthalmitis occurs by introduction of microorganisms into the eye from trauma or surgery.[15-17,20,43-46] It can also be the end result of preexisting scleritis or keratitis.[15,16] *Mucoromycotina* infection in the surrounding soft tissue and cryptococcal neuroretinitis may also lead to exogenous endophthalmitis (Table 1). Patients with exogenous FE are rarely immunocompromised.[18,19] Jones was one of the first ophthalmologists who noticed on exogenous FE in 70s and summarized the clinical experience in 25 cases, 9 (36%) were cases of exogenous FE.[47] Visual acuity outcomes were poor in these 9 eyes, with 7 (78%) being enucleated or eviscerated. There are two more recent papers by Pflugfelder et al[18] and Wykoff et al[19] describing large series with exogenous FE. We will analyze the united information of these two studies, since they are similar in most aspects. Total number of cases was 60 (19+41); 25 of which associated with keratitis (41.6%), 19 with surgery (32%) and 16 with trauma (26.6%). The proportion of fungal isolates were also similar in both studies; most of them being molds (86.6%) and 13.3% being yeasts.

Exogenous FE may have a period of latency of weeks to months before clinically detectable disease occurs. Even then the infection is often confined to the anterior chamber, pupillary space, or anterior vitreous. However, there is a report of a series of 5 patients with relatively early onset (10-62 days) *Aspergillus* endophthalmitis following cataract surgery.[46]

Antifungal Agents	Systemic Doses	Intravitreal Doses
Polyenes		
Amphotericin B	0.6-1 mg/kg/day IV	0.005-0.01 mg/0.1 ml
Azoles		
Fluconazole	400-1600 mg/day PO or IV	Experimental
Itraconazole	400-800 mg/day PO or IV	-
Voriconazole	6 mg/kg/day PO or IV	0.1 mg/0.2ml
Posaconazole	400-800 mg/day PO or IV	-
Echinocandins		
Caspofungin	70 mg loading dose, 50 mg/day IV	0.1 mg/0.1 ml
Micafungin	50-150 mg/day IV	-
Anidulafungin	50-100 mg/day PO or IV	-

Table 2: Systemic and intravitreal antifungal agents used for fungal endophthalmitis.[35] (PO: per oral, IV: intravenous)

Diagnosis of exogenous FE is mostly possible with intraocular fluid cultures which may be positive in most of the cases.[19] Treatment of exogenous FE usually starts with intraocular (intracameral ± intravitreal) amphotericin B other than systemic treatment (Table 2). Other primary antifungal treatments may be intravitreal voriconazole or miconazole. Oral and subconjunctival antifungal agents may also be added to the treatment especially in the keratitis-associated patients. Systemic antifungal agents include fluconazole, ketoconazole, voriconazole, itraconazole, amphotericin B and miconazole which are especially important in immunocompromised patients. Pars plana vitrectomy (PPV) would be the best treatment option in eyes unresponsive to medical treatment. Pflugfelder et al[18] and Wykoff et al[19] have reported that, approximately 90% of the cases received intraocular amphotericin B and 61-84% of the eyes had to have PPV.

NB
While preparing the intravitreal doses of amphotericin B, it is important to use distilled water or 5 % dextrose NOT saline.

Prognosis of exogenous FE depends on the subgroup of etiology. A final vision of 20/400 or better was achieved in 54% of eyes and almost all were in the keratitis or the postoperative groups.[18] Conversely, although 24% of the eyes were enucleated, most of these were among the open-globe patients. Final visual outcomes seem to be

variable, with the open-globe–associated patients having the poorest outcomes. Overall, the prognosis in recent papers is becoming better with 44% of patients reaching a final visual acuity of 20/80 or better.[18,19] This improvement in the results may because of the increased and earlier recognition of the disease itself and the resistance of the disease to the antifungal agents used and skipping to the alternative antifungal agents earlier in the clinical course, when resistance is suspected.

3.2. FUNGAL KERATITIS

Fungal keratitis (FK) is the most frequent fungal infection of the eye.[5,7] FK is one of the major causes of blindness especially in Asia. Most of the reports with large series of FK are originated from India.[21,25-27,47,48] But there are reports from other countries.[50] There are three major predisposing factors for FK; trauma with organic or vegetable matter, use of contact lenses, pre-existing systemic conditions and ocular surface problems. Trauma is the key predisposing factor, in healthy young males engaged in agricultural or other outdoor work. There is a history of trauma in more than 5 to 65 % of FK cases and trauma was identified as a principal risk factor in 44 % of children who had microbial keratitis in southern United States.[27,28,49] Trauma related keratitis is mostly filamentous keratitis. Abrasions caused by contaminated contact lenses (especially hydrophilic contact lenses) may predispose to *Fusarium* keratitis.[20-26] Insufficient tear secretion, defective eyelid closure, pre-existing epithelial defect, refractive surgeries, herpes keratitis, allergic conjunctivitis, use of eye drops (especially steroids) and systemic problems like diabetes mellitus, immunosuppression may predispose to keratitis mostly associated with *C. albicans* and related fungi.[20-28,49,51]

Filamentous fungi form the major etiologic agents of FK. *Fusarium* species (37- 62%) and *Aspergillus* species (24–30%) have been implicated as main pathogens (Table 1). Other less frequent isolates have been listed in Table 3.[28] Yeast like fungi are supposed to be rare pathogens for keratitis (0.7%),[24] however, there is only one series from Wills Eye Hospital reporting the *C. albicans* as the most common pathogen (45.8%) causing keratitis.[51] Figure 6 shows a case of *Candida* keratitis associated

with penetrating keratoplasty.

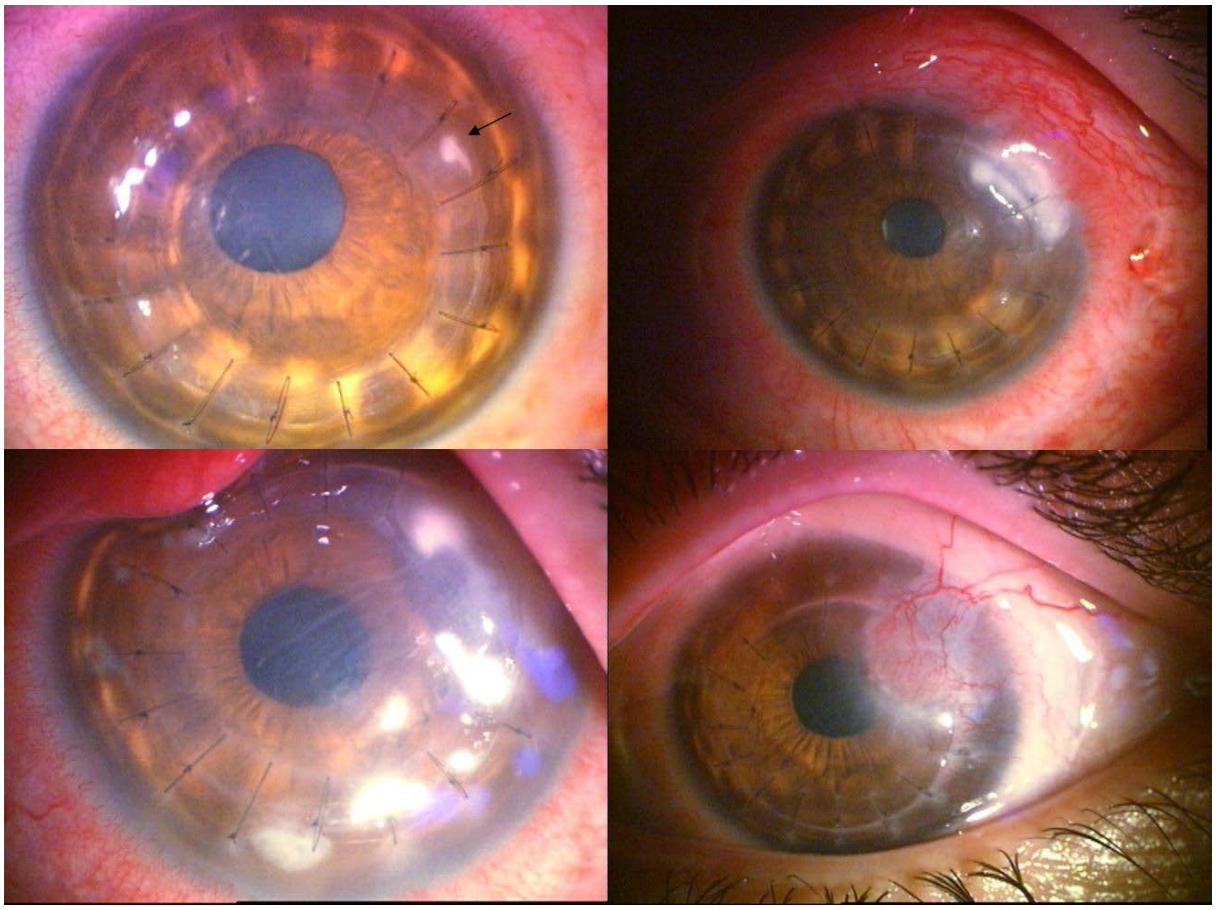

FIGURE 6. *Candida* keratitis associated with penetrating keratoplasty. Topleft figure shows early infiltration at 2 o'clock position of donor-hostcornea border (arrow). The infiltration is getting larger (top right) involving other parts (lower left) in spite of antifungal treatment. Lowerright figure shows the vascularized scarring at the end of 6 months. (Courtesy of Fikret Akata, MD).

Filamentous fungi	
Main Pathogens:	*Fusarium (F. solani, F. oxysporum)*
	Aspergillus (A. fumigatus, A. flavus)
Others:	*Scedosporium (S. apiospermum)*
	Penicillium (P. spinulosum, P. citrinum)
	Acremonium (A. potronii, A. kiliense)
	Curvularia (C. lunata, C. geniculata, C. senegalensis)
	Bipolaris (B. spicifera, B. hawaiiensis)
	Exserohilum (E. rostratum, E. longirostrata)
	Coelomycetes (Lasiodiplodia, Colletotrichum)
Yeasts	
Candida species (*C. albicans*)	

Table 3: Fungi causing keratitis. (Adapted from Srinivasan M)[28]

Clinical presentation of FK may vary depending on the etiologic agent; however, the most common lesion is indolent and dry, with a leathery, tough, raised surface. The corneal defect usually becomes apparent within 24 to 36 h after the trauma. Symptoms are usually nonspecific, although possibly more prolonged in duration (5–10 days) than in bacterial ones. Feathery borders or hyphate edges are seen in 70% of patients, and satellite lesions in 10% of patients, with FK. Hypopyon is present in 55 % of cases.[50] There is minimal to absent host cellular infiltration. When there is an infiltrate, it is often surrounded by a ring, which may represent the junction of fungal hyphae and host antibodies. Descemet's membrane is impermeable to bacteria but can be breached by fungal hyphae, leading to endophthalmitis.[20] Figure 7 shows clinical pictures and hyphal invasion of corneal tissue in one of our cases with fungal keratitis.

Since many of the filamentous fungi grow slowly, the disease often remains unrecognized and untreated for days or weeks until growth is visually detected, and this delay may contribute to a poor response to therapy. Early recognition of the disease is crucial to facilitate a complete recovery. Identification of the pre-existing ocular and systemic diseases usually helps to prevent the misdiagnosis.

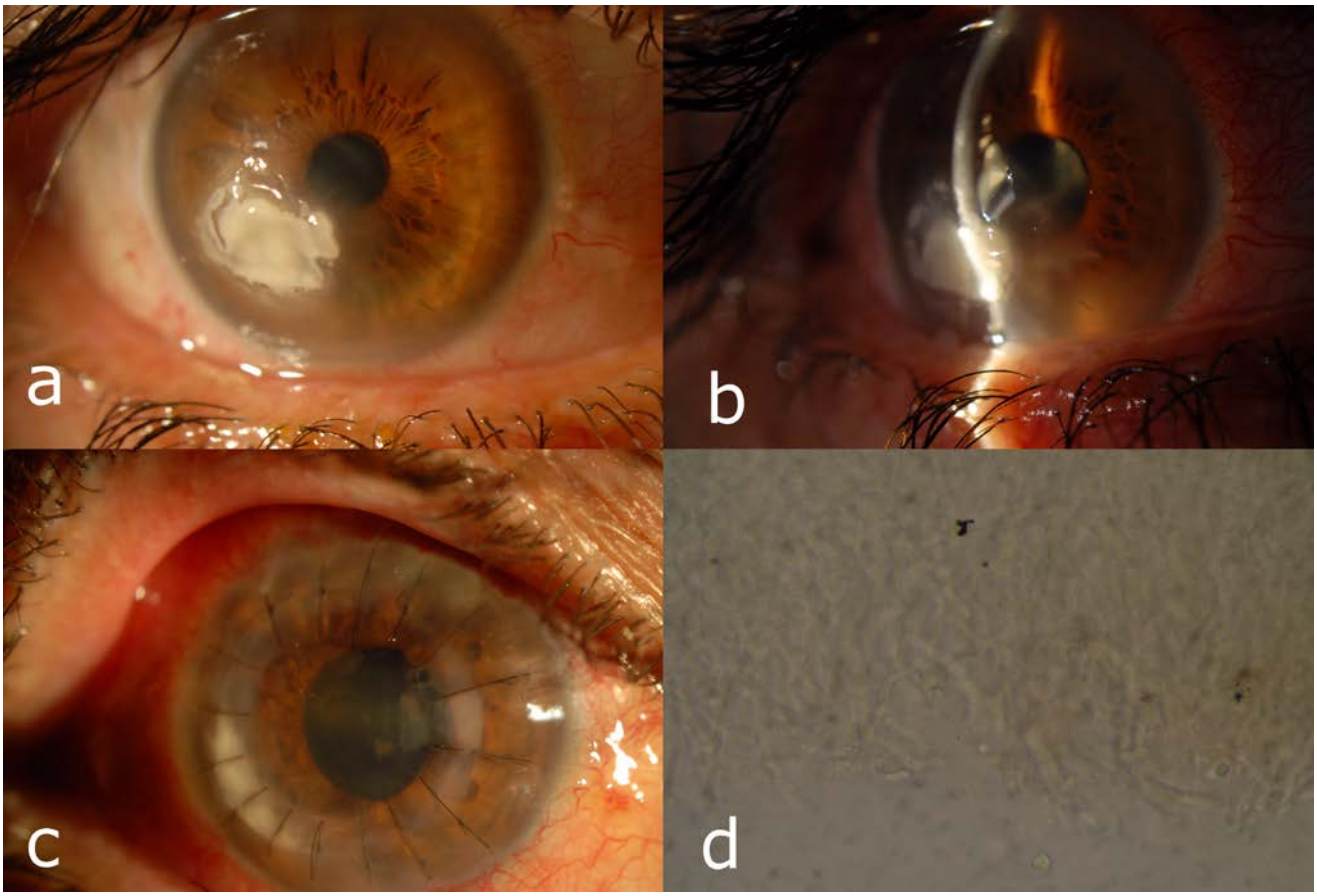

FIGURE 7. Elevated corneal lesions with grey/white surface, with a ring infiltrate (a). Despite topical and intrastromal amphotericin B and voriconazole treatment stromal infiltration progressed and hyphae reached to anterior chamber (b). After therapeutic penetrating keratoplasty (c). Microscopic visualization of fungal hyphae (d).

NB
Debridement of the lesion is performed every 24-48 hours and works by debulking organisms and necrotic material and by enhancing penetration of the antifungal drug.

Treatment of fungal keratitis is reviewed in Table 4. Topical natamycin (5 %) or amphotericin B (0.15 %) is usually the first-line therapy for superficial keratitis. These two drugs are called as polyenes. The drug of choice will be topical natamycin if hyphae are definitely seen by microscopy, on the other hand, it will be topical amphotericin B or topical fluconazole if yeasts or pseudohyphae are seen on microscopy.[25,52] Repeated debridement of the epithelium helps the drugs to penetrate deeper in the cornea. Topical therapy is usually applied hourly for several days and the frequency of application is then gradually reduced. A large prospective study on culture positive 115 FK cases treated with 5% natamycin monotherapy revealed that, predictors of treatment failure were ulcers that exceeded 14 mm, the presence of hypopyon, and identification of *Aspergillus*. In other words, predictors of poor outcome in FK treated with 5% natamycin monotherapy were larger ulcer size and infection with *Aspergillus*.[53] Deeper and larger lesions need some form of systemic therapy, such as subconjunctival or intravenous miconazole, oral ketoconazole, itraconazole, fluconazole or voriconazole all of which are in the group of azole compounds.[25-28] Intracameral amphotericin B may be another option for these cases. Penetration characteristics of systemic drugs should be compared for the management of ocular infections.

NB
In cases with deep fungal keratitis recalcitrant to topical therapy alone, intrastromal injection of antifungals (voriconazole: 50µg/0.1mg, amphotericin B: 5-10 µg/0.1ml) have also been used successfully.

This phenomenon may attribute treatment approaches.[54] If medical therapy fails to control the infection, surgery should be considered to save the eye and visual function before the progression of the disease to the peripheral cornea. N-butyl cyanoacrylate tissue adhesive can be used in the management of corneal thinning or perforation associated with active FK which can lead to resolution of infiltration with scar formation in 63% of the eyes.[55] Amniotic membrane transplantation may also help in promoting healing. Penetrating keratoplasty is the ideal method to treat nonhealing FK threatening perforation. Structural integrity and eradication of sepsis is achieved in up to 90% of eyes with lower graft clarity rates.[56] Figure 8 shows a FK case with corneal perforation treated with penetrating keratoplasty. The patient in Figure 9 needed combined surgery for FK and fungal endophthalmitis.

Antifungal drugs are not always effective in severe keratomycosis. In some cases, corneal transplantation is required as the only alternative after ineffective chemotherapy. Collagen cross linking (CXL) is a new tool in the management of infectious keratitis resistant to antimicrobial treatment. At the begining of 2000, CXL was first used for the treatment of patients suffering from melting ulcer of the cornea of various origins. This method indeed, was developed to increase the biomechanical strength of the cornea and to stop the progression of keratoconus. CXL is based on using riboflavin as a photosensitizer, which generates reactive oxygen species when activated by UV-A. During the CXL procedure, drops of 0.1 % riboflavin solution in 20 % dextran are instilled onto the cornea every 5 min for 30 min. After allowing riboflavin to permeate through the cornea and appear in the anterior chamber, the cornea is exposed to ultraviolet A (UVA) light with a wavelength of 370 nm and an irradiance of 3 mW/cm2 for a total time of 30 min. New treatment option was generated to be used in resistant keratitis cases.[57]

In a recent study, Sun et al., showed that the UVA (365 nm) / riboflavin mediated CXL has anti-fungal effect and the inactivation ratio of CXL increases along with the decrease of the cell concentration for *C. albicans* and *F. solani*.[58] However, there are

other *in vitro* studies showed mixed results especially for fungal keratitis. Also, clinical reports are inconsistent and difficult to interpret.[59,60]

The expected complications of CXL in infectious keratitis are endothelial cell loss related to fungal deep infiltration and reactivation of previous Herpes simplex infection. With the intention of avoiding these complications, it could be proposed that the previous history of Herpes infection should be excluded. After all, CXL should be considered in cases of severe unresponsive infectious keratitis before undertaking emergency keratoplasty.[61]

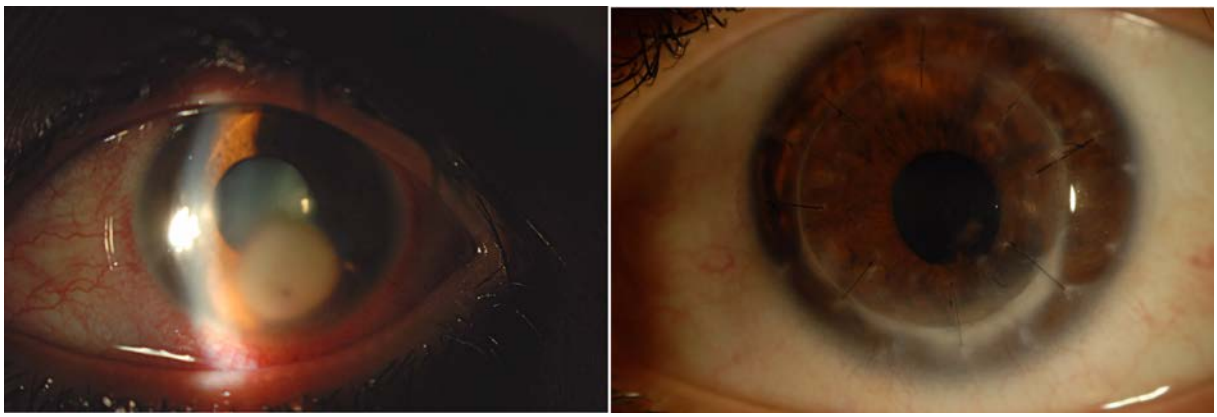

FIGURE 8. Right eye of a 19 year old female patient is seen. Trauma with a rose bush is learned from history. Indolent, dry ulcer with feathery edges is seen. Despite treatment with topical and intrastromal amphotericin B, disease progression leads to perforation. Left figure shows indolent and dry ulcer with a perforation in the center. There is a satellite lesion lateral to the ulcer and breach of Descemet's membrane is seen. Figure on the right side shows after succesful therapeutic penetrating keratoplasty.

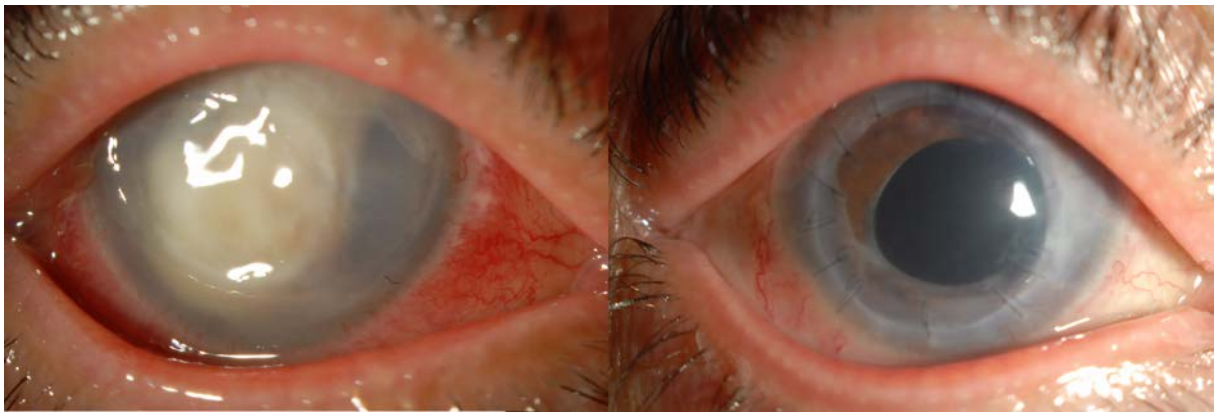

FIGURE 9. A patient who is referred late shows *Candida* keratitis with endophthalmitis. Despite intravitreal and intracameral antifungal injections surgical intervention was needed. The figure on the right shows clear graft after successful penetrating keratoplasty, lensectomy, and pars plana vitrectomy.

Antifungal Agents	Topical	Intracameral	Subconjunctival	Intravitreal	Oral	Intravenous
Polyenes						
Amphotericin B	1.5-5mg/ml	5-10µg/0.1ml		5-10µg/0.1ml		0.5-0.7 mg/kg/d
Natamycin	50mg/ml					
Azoles						
Ketaconazole					200-400 mg/d	
Fluconazole	2mg/ml				100-400 mg/d	200-400 mg/d
Itraconazole	10mg/ml			0.005 mg/0.05 ml	200-400 mg/d	200 mg/d
Voriconazole	1mg/ml			0.05-0.2 mg/0.2ml	200 mg twice daily	3-6 mg/kg twice daily
Posaconazole	100mg/ml				200 mg three times daily	400-800 mg/kg/d
Econazole	20mg/ml					
Miconazole	10mg/ml	5 mg/0.5 ml	1.2-10mg/ml	0.025-0.05 mg/0.1 ml		
Pyrimidines						
5-Fluorocytosine					25-37.5 mg/kg/d four times daily	
Allylamines						
Terbinafine					250 mg/d	
Echinocandins						
Caspofungin	1.5-5mg/ml			0.1 mg/0.1 ml		50 mg/d
Micafungin	1mg/ml					

Table 4. Classification and doses of antifungal agents used for fungal keratitis. (d: day, modified from Alfonso et al.[25])

3.3. ORBITAL FUNGAL INFECTIONS

Infections of the orbit usually occur as a secondary process from the surrounding structures, such as the paranasal sinuses, skin, brain, and the nasopharyngeal cavity. The inflammatory conditions that affect the eyelids and the orbit are broadly divided into preseptal (periorbital) and postseptal (orbital) cellulitis. There are, however, some other entities that are grouped within the orbital infection group. The current classification of orbital inflammation was proposed by Smith and Spencer[62] and later modified by Chandler et al.[63] They classified orbital inflammation in 5 groups: group 1 for preseptal cellulitis; group 2 for orbital cellulitis; group 3 refers to a subperiosteal abscess; group 4 classifies a diffuse orbital abscess; and group 5 refers to cavernous sinus thrombosis.

Orbital cellulitis is most commonly caused by bacterial infection. Fungal and viral etiologies occur less frequently. Mycotic orbital cellulitis is seen in patients with uncontrolled diabetes mellitus or other immunocompromised states such as AIDS, malignancy or steroids use.[64] They may be invasive or non-invasive. Fungal etiologies include Mucoromycotina (Formerly Zygomycetes) (*Mucor*, *Rhizopus* and *Lichtemia*, formerly *Absidia* spp.), *Aspergillus* spp., and to less extend *Blastomyces*, *Sporothrix* spp and *Bipolaria* spp.[64] Invasive *Aspergillus* and Mucoromycotina infections have a marked predilection for the orbit and the paranasal sinuses.

3.3.1. Orbital zygomycosis

Revision has been made in the fungal taxonomy. Fungal kingdom re-classified into four orders, one is Glomeromycota, containing subphylum named Mucoromycotina. Zygomycetes positioned under subphylum Mucoromycotina. Although mucormycosis is the term used to refer to fungal infections of this class, the correct term is zygomycosis. *Mucor* and *Rhizopus* are two genera of the order *Mucorales*, a subset of the class Zygomycetes. They are saprophytic fungi that are normally not pathogenic to humans. In immunocompromised states, the inhaled spores of Zygomycetes, which are normally eliminated with phagocytosis, start to progress through the nose to maxillary sinuses, ethmoids and orbit. Spread from paranasal sinuses to orbit is usually via the nasolacrimal duct and medial orbit. The thinness of lamina papyracea and perforation of

medial wall by blood vessels are the causes of spread through the medial wall. Infection enters to central nervous system (CNS) through the orbital roof, apex and cribriform plate. Organism invades blood vessel walls, causing necrosis, thrombosis, obstruction and ultimately infarction of involved tissues. Internal carotid, middle cerebral, ciliary and retinal arteries as well as cavernous sinus are all subject to this progression. Practically all conditions causing immunosupression may be a predisposition to these infections. In a recent global clinical registry study, the most common underlying conditions in 41 cases of invasive zygomycosis were malignancies (63.4%), diabetes mellitus (17.1%) and solid organ transplantation (9.8%).[65] Rhino-orbito-cerebral (ROC) infection produces characteristic clinical features of low grade fever, periorbital pain, headache, lethargy, sinusitis, unilateral facial swelling, black nasal and palatal eschar, decreased vision, afferent pupillary defect, proptosis, and ophthalmoplegia.[67-70] CT and MR imaging are often used in the diagnostic work-up; however, CT findings are nonspecific.[71] Biopsy material is crucial for the histopathological work-up. Figure 10 shows a case of orbital zygomycosis diagnosed by histopathological findings.

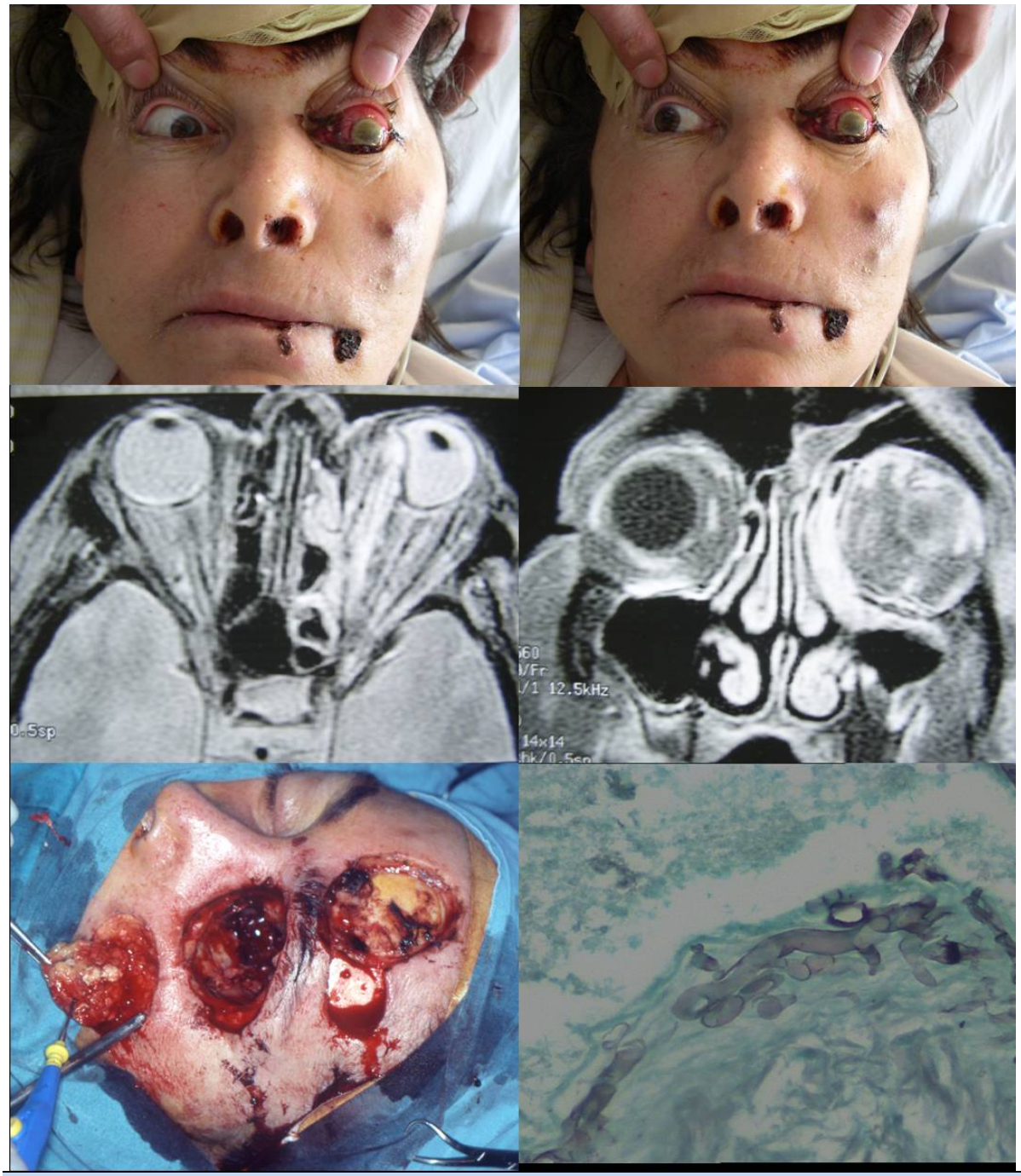

FIGURE 10. 58 year old lady with diabetic ketoacidosis had total ophthalmoplegia with no light perception, left facial nerve palsy, left hemifacial pain, necrotic black skin lesions over the forehead, cheek and lip. Ethmoidal sinusitis was apparent in MRI films. Surgical debridement of all necrotic tissues together with exanteration of the orbit was performed in addition to intravenous amphotericin B treatment. Histopathological examination of the tissues revealed broad, non-septate hyphae resembling Zygomycetes infection (GMS 100x). (Courtesy of Onur Konuk, MD)

There is need for a high index of clinical suspicion for early diagnosis. Control of the underlying predisposing illness along with the timely medical and surgical treatment proves extremely important for prognosis. The combined modality of early surgical debridement and antifungal agents is used for treatment of ROC infection.[72] Parenteral antifungal treatment with liposomal amphotericin B is the main medical treatment. Surgical treatment is mostly aggressive including orbital exenteration and pansinusectomy with endoscopic sinus surgery; however, timely limited surgical intervention without exenteration may be successful in early and limited cases. Prognosis of the disease is still poor, not only because of the aggressive nature of the disease, but also because of the delayed diagnosis.[73]

Table 5 summarizes the management of zygomycosis.

Early diagnosis of the infection
Correction of underlying predisposing disease conditions
Treatment of the co-existent bacterial infection
Surgical debridement of the necrotic tissues and getting biopsy material
Microbiologic examination of the biopsy material
Intravenous antifungal agents (Amphotericin B: 0.6-1 mg/kg/day)

Table 5: Management of Zygomycetes infections.

3.3.2. Orbital Aspergillus Infections:

Aspergillosis is the most common cause of fungal sinusitis and orbital fungal infections. *Aspergillus* is also a saprophytic fungus that is normally not pathogenic to humans. Usual entry site is through the nose and paranasal sinuses like Zygomycetes. The infection has a predilection for the immunocompomised host, especially in cancer (leukemia and lymphoma) patients. Many different orbital presentations by *Aspergillus* occur even in the healthy host. These infections are not invasive, and drainage or excision may lead to clinical resolution.[74]

Clinical presentations of orbital invasive aspergillosis are similar to other inflammatory

orbital diseases and neoplastic diseases. Invasive disease in the compromised host may begin as dacryocystitis, masquerade as an optic nerve tumor,[75] or present as an entirely retrobulbar process such as in our case showed in Figure 8. Orbital disease with *Aspergillus* in the immunocompromised host may also begin as sphenoid and/or ethmoid sinusitis with erosion of the bony orbit, leading to invasion of the orbital space and proptosis. Proptosis may be the initial sign of fungal sinusitis even in immunocompetent individuals.[76,77]

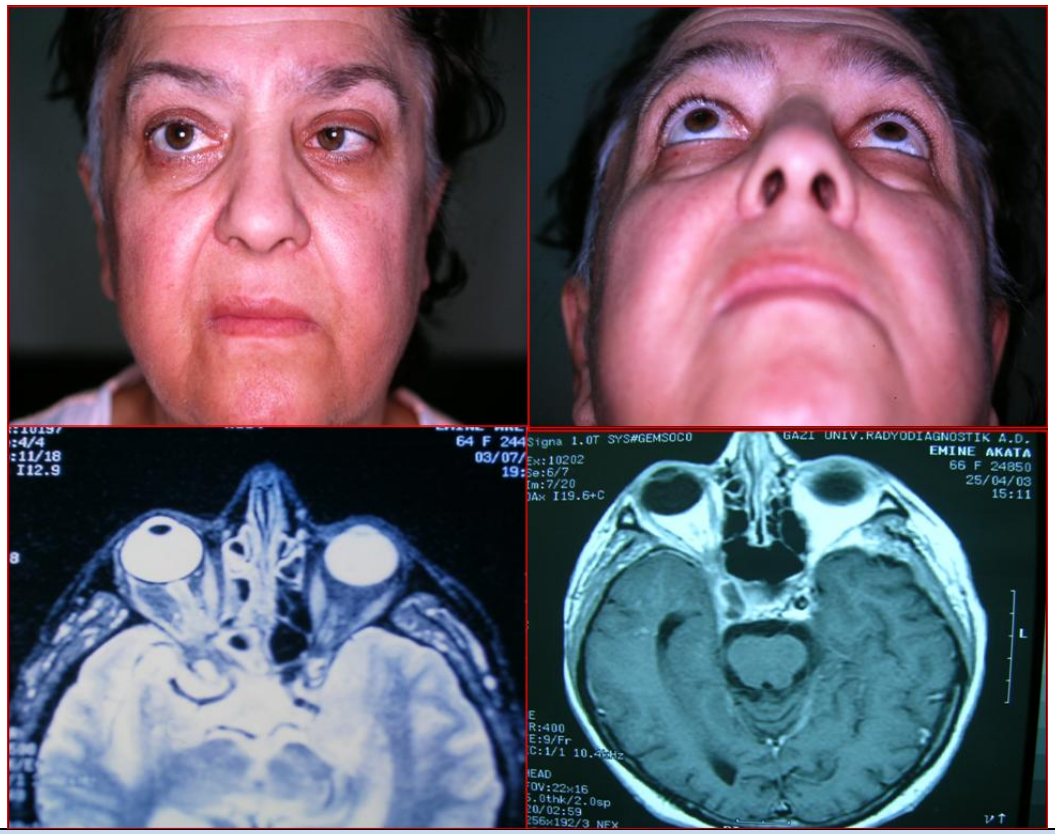

FIGURE 11. 65 year old diabetic lady with total external painful ophthalmoplegia, proptozis and vision loss in the right eye for the last 2 months. Orbital MRI examination revealed ethmoidal and sphenoidal sinusitis and inflammatory reaction extending around the optic nerve and orbitalapex (lower left).Microbiologic examination of the endoscopic sphenoid sinus biopsy material revealed septated hyphaeand the colony of *Aspergillus fumigatus*. Treatment started with intravenous liposomal amphotericin B, local debridment and irrigation of the involved areas with amphotericin B and continued with oral itraconazole for 3 months which resulted in total resolution of ophthalmoplegia and inflammatory findings in MRI (Lower right). (Courtesy of Onur Konuk, MD)

High rates of negative biopsy results have been reported, especially because the fungus appears only in late-stage clinical samples. Therefore, if diagnosis is not made on the first biopsy, and fungal infection is still suspected, a second biopsy should be performed, especially before considering treatment with corticosteroids. CT or MRI of the sinuses, orbit, and brain are important in diagnosing this condition, determining the extent of disease and in planning the surgical approach.

Treatment is similar to Zygomycetes infections (Table 5). Management often begins with surgical debridement followed by systemic antifungal drug therapy. Some antifungals are used, such as polyenes (amphotericin) and azoles (itraconazole and voriconazole), and other newer classes such as lipid complex nystatin and echinocandins.[78] Among them, amphotericin B is a conventional drug for treatment of invasive aspergillosis. Newer formulations, including lipid complex and liposomal forms, have been developed to decrease the toxicity of amphotericin B and indeed seem to be less toxic. Data from various sources suggest that response rates to the different drugs are only 40 % to 60 %.[79] Of the azole class, itraconazole and voriconazole are promising and are safer and easier to administer than amphotericin B. Orbital invasive aspergillosis is often fatal with a mortality rate up to 40-50 %.[74] Poor prognostic factors are reported to be associated with delayed and incorrect initial diagnosis, presence of fever, intracranial extension of infection, and histopathology demonstrating hyphal invasion in blood vessels or adjacent tissue.[77-80]

4. COLLECTION AND TRANSPORT OF SPECIMENS

Ocular samples should be obtained in all cases suspected with fungal infections. Many different microbes may enter the eye following ocular trauma and can cause post-traumatic endophthalmitis. Anterior chamber fluids may be aspirated through the limbus using a needle. Vitreus specimens are obtained through the pars plana. Vitreus fluid should be placed in sterile containers. Anterior chamber taps and vitreous taps are collected in most of the cases. Systemic blood cultures should also be obtained simultenously in case of endophthalmitis. Intraocular samples should be inoculated onto agar plates immediately. On side inoculation is preferable.[80,81] Ocular samples should be collected aseptically to avoid microbial contamination. If needle biopsy is unproductive, samples should be obtained by vitreus biopsy or vitrectomy when allowed by the general condition of the patient. If progressive or severe vitritis is noted, both aqueous and vitreus cultures should be obtained for microbiologic study. Specimens collected aseptically, placed in sterile containers, delivered to the laboratory within 2 hours, processed, and then inoculated to primary isolation media within a few hours of collection. Viability may decrease with prolonged specimen storage. Swabs are not encouraged for ocular sampling. Specimens should be transported in a sterile, humidified, leak-proof container. Specimens should be processed and inoculated to primary isolation media as soon as possible after collection, ideally within a few hours. It should not be assumed that succesful methods to storage of fungal cultures are suitable for temporary storage of clinical specimens that harbor relatively few fungal cells. The effect of refrigeration on fungal specimens has not well-studied, but if processing is to be delayed for more than several hours, it is recommended that specimens be stored under refrigeration at 4^0C with following exceptions; blood and vitreous fluid are stored at 30^0-37^0C; swab specimens are stored at 15^0-30^0C. Collection and transport directories regulations should be shared with clinicians and make part of the instructions for collection and submission of specimens distributed to staff.

In general patients with keratitis undergo corneal scraping for direct examination,

culture and molecular methods. Gram staining and potassium hydroxide (KOH) preparation are routinely performed. [82] Material for microscopy and culture is obtained by scraping the base and edges of the ulcer with a sterile blade or spatula several times. Sometimes it may not be possible to obtain corneal scrapes because of the occurrence of a very small or nonexistent epithelial defect. In such situations, corneal material may be obtained by performing corneal biopsy, corneal material may also be obtained at the time of performing a penetrating keratoplasty.[83] Table 6 summarizes the specimen collection and the processing, and Table 7 summarizes the specimens used for diagnosis of ocular fungal infections.

Specimen	**Collection**	**Undesirable specimens**	**Processing**	**Media**
Cornea	Corneal tissue in 1.0 ml sterile distelled water.	Dried specimen	Direct	SDA, sheepblood agar plate
Eye fluid	In collection tubes, or filtered fluid on filter paper	Swabs	Concentrate fluids, divide filter	SDA, IMA, BHI, sheep blood agar plate

Table 6: Specimen collection for ocular samples

Infection	Specimen Type
Orbital lesions	Biopsy specimens Purulent material aspirated Serum for serological investigations
Blepharitis and eyelid lesions	Cotton swabs Lid biopsy samples
Dacryoadenitis	Lacrimal gland biopsy samples
Dacryocanaliculitis	Purulent material
Dacryocystitis	Lacrimal sac material
Conjunctivitis	Scrabbed lesion Conjunctival biopsy specimen
Keratitis	Swabs of lid and conjunctiva Corneal scrapes Biopsy specimens
Scleritis	Same as conjunctivitis or keratitis If abscess is present, aspirated material Scleral biopsy
Endophthalmitis	Conjunctival swab Vitreous or aqueous aspirate Vitreous biopsy specimen Vitreous wash material
Choroiditis and retinitis	Recovery of fungi from blood or other body lesions Immunologic tests for antigens Rarely, material is collected from the lesion itself by surgery

Table 7: Specimens used for diagnosis of ocular fungal infections. (Adapted from Thomas PA)[7]

5. LABORATORY DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS

5.1. Conventional microbiologic techniques

5. 1. 1. Direct Microscopy (Gram, Giemsa, Calcoufluor Stains)

5. 1. 2. Culture

5. 1. 3. Identification

5. 1. 4. Susceptibility Testing

5. 2. Histopathologic techniques

5. 2. 1. Conventional microscopy

5. 2. 1. 1 Routine stains (H&E)

5. 2. 1. 2. Special stains (GMS, Mucicarmine, PAS)

5. 2. 2. Direct immunofluorescence

5. 2. 3. In situ hybridization

5. 3. Immunologic techniques

5. 3. 1. Cryptococcal antigen test

5. 3. 2. Antigen test for dimorphic agents

5. 3. 3. Galactomannan test

5. 3. 4. Mannan test

5.4. Biochemical techniques

5. 4. 1. Metabolites (D-Arabinitol)

5. 4. 2. Cell wall components (Beta- glucan)

5. 5. Molecular techniques

5. 5. 1. PCR for direct detection of pathogen

5. 5. 2. Molecular methods for the identification of fungi

5. 5. 3. Strain typing with molecular methods

5. 6. Others

Abbreviations: H&E, hematoxylin and eosin, GMS, Gomori's methanamine silver, PAS, periodic acid-schiff

5.1. CONVENTIONAL MICROBIOLOGIC TECHNIQUES

The prompt diagnosis of mycoses requires a high index of suspicion and an appreciation of specific risk factors that may predispose a patient to ocular fungal infections. Determination of the identity of the specific etiological agent of mycotic disease is very important for the therapeutic considerations. For example using azoles or, amphotericin B is inadequate for many fungal infections. Classical diagnosis of fungal infections depends on direct microscopic examination or staining of tissue sections and the isolation of the fungus in culture. Tests for the detection of antibodies and antigens, metabolites and fungus specific nucleic acids and other methods such as confocal microscopy have great appeal. Radiographic imaging of the orbit and paranasal sinuses is invaluable for both the initial evaluation and for monitoring disease progression and response to treatment of sinoorbital disease.[83,84]

5. 1. 1. Direct Microscopy (Gram, Giemsa, Calcoufluor Stains)

Direct microscopic examination of specimens is generally considered to be among the most rapid and cost-effective means of diagnosing ocular fungal infections. Most of organisms that can be specifically identified by direct microscopy, because they possess a distinctive morphology. Microscopic examination of a KOH preperation can reveal the presence of fungal structures. The purpose of the KOH is to dissolve the human cells, allowing visualization of the fungi. The specimen is either treated with 10% KOH to dissolve tissue material, leaving the alkali resistant fungi intact, or stained with special fungal stains. Typical yeast cells or spherules can provide an infections due to *H. capsulatum*, *B. dermatitidis*, *C. neoformans*, *C. immitis* complex. Microscopic detection of fungal elements in tissue can assist the laboratory in selecting the most appropriate means to culture the specimen and also is helpful in determining the significance of culture results. The latter is especially true when the organism isolated in culture is a known component of the normal flora or is frequently found in the enviroment. For example, the presence of non-septate hyphae of zygomycetous fungi should prompt the use of malt agar or even sterile bread without preservatives for its isolation. Direct

microscopy is less sensitive then culture and a negative direct examination does not rule out a fungal infection. Gram and giemsa stains are most commonly used techniques to demonstrate the presence of microorganisms in clinical specimens. Calcoflour white stains the cell wall of fungi causing the fungi to flourescence for easier and faster detection. The Gram stain is usefull for the detection of *Candida* and *Cryptococcus* spp and also stains the hyphal elements of moulds such as *Aspergillus*, the Zygomycetes and *Fusarium* spp. Many fungi will stain blue with the giemsa stain, but this stain is especially useful for detecting yeast of dimorphic forms. Filamentous fungi show hyaline, branching, septate hyphae in clinical samples. In contrast, dematiaceous fungi show pigmented hyphae, wheras zygomycetes characteristically show broad, ribbon-like, aseptate or sparsely septate hyphae. Fontana-Masson stain for melanin can be used for the visualization of dematiaceous fungi in ocular samples. Stains such as hematoxylin and eosin (H&E), gomori methanamine silver GMS and periodic acid-schiff (PAS) are used for detection of fungi in cytologic preperations. H&E can be visualize all fungi but some of them may be missed. GMS and PAS stains are more fungus specific stains which allow the detection of small numbers of organisms and for clearly defining characteristic features of fungal morphology.[84-86]

5. 1. 2. Culture

The most sensitive method for diagnosing fungal infections is the isolation of the infectious agent on culture media. Culture is necessary to identify the fungi and if indicated, to determine the in vitro susceptibility to various antifungal agents. No single culture medium is sufficient to isolate all fungi, and it is generally accepted that at least two types of media, selective and nonselective be used. Interpretation of the results of fungal cultures may be difficult due to the colonization of body sites and contamination of specimens or cultures by enviromental organisms, many of which can also serve as etiologic agent of opportunistic mycoses. The isolation of dimorphic pathogens (*H. capsulatum, B. dermatitidis, C. immitis*) are virtually always considered to be clinically significant. The clinical significance of isolation of filamentous fungi from cultures may be confirmed upon direct microscopic visualization of the organism in viable tissue.

Fungi grow in most media used for bacteria, however, growth may be slow, and a more enriched medium such as brain heart infusion (BHI) agar, or Sabouraud dextrose agar (SDA) is recommended. Cycloheximide is often added to this medium in order to inhibit contaminants many opportunistic pathogens are susceptible to cycloheximide, thus one should always pair cycloheximide containing media with complementary media without cycloheximide.

Once inoculated, fungal cultures should be incubated in air at a proper temperature and for a sufficient period of time to ensure the recovery of fungi from clinical samples. Most fungi grow optimally at 25^0C to 30^0C although most species of yeasts grow well at 35^0C to 37^0C. Specimens should be incubated for two weeks minimum, mostly four weeks is required for negative culture result.[84-87]

5. 1. 3. Identification

Identification of fungi to genus and species level is necessary to optimize therapeutic considerations. Distinguishing yeasts from moulds is the first step in mycology practise. Colony morphology are usually provides a reliable evidence, but microscopic examinationis required for the confirmation. Additional biochemical and physiologic testing are required for distinguishing one yeast from others. Definitive identification of moulds is based on its microscopic morphology, whereas the identification of both yeasts and moulds may be enhanced by specialized immunologic and molecular techniques.

Identification to genus and species, depending on the fungus, requires more detailed microscopic study to characterise structure.The most simple grouping, based on morphology, lumps fungi into either yeasts or moulds. A yeast can be defined morphologically as a cell that reproduces by budding or by fission (Figure 12).


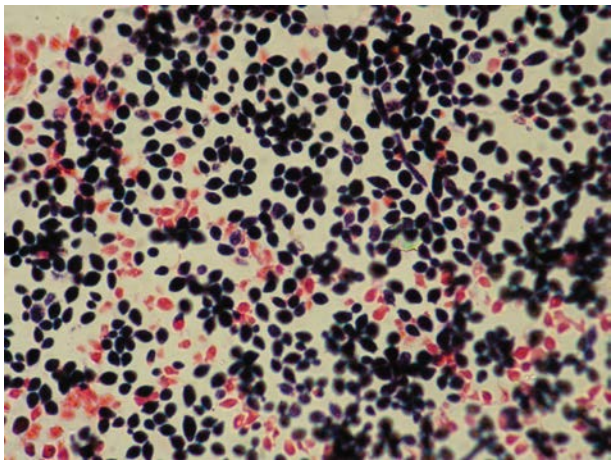

Figure 12. Yeast morphology; **12a.** Macroscopy of *Candida* colony, **12b.** Microscopic morphology by Gram stain of *Candida* colony (40x).

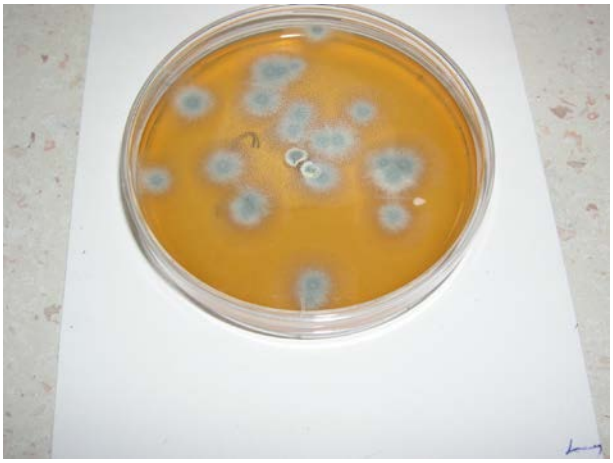
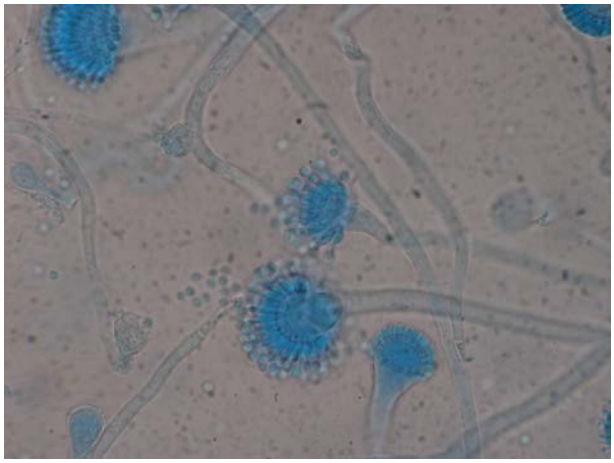

Figure 13. Mould morphology; **13a.** Macroscopy of *Aspergillus* colony, **13b.** Microscopic morphology by Lactophenol cotton blue preparation (40x).

Yeasts are usually produce round, pasty or mucoid colonies on agar plates. Moulds on the other hand, are multicellular orgranisms consisting of threadlike tubular structures called hypha (Figure 13). The aerial hypha produce specialized structures known as conidia. Yeast identification requires additional biochemical and physiologic testing. However, the definitive identification of a mould is based almost entirely on its microscopic morphology.[84-88] Table 8 summarizes the basic principles of fungal identification procedures and the main characteristics of fungi.

FUNGUS	Microscopic Morphologic Features in Clinical Specimens	Characteristic Morphological Features in Culture		Additional Tests for Identification
		Macroscopic	Microscopic	
Candida	Oval budding yeasts 2-10 μm in diameter. Pseudohyphae may be present.	Yeast colonies are pasty, creamy, white and opaque	Blastoconidia, pesudohyphae, Chlamydospore in some species.	Carbohydrate assimilation. Morphology on corn meal agar.
Aspergillus	Septate, dichotomously (45^0) branched hyphae of uniform width (3-6 μm)	Mould colonies are blue-green, yellow-green, or black and velvety, cottony.	Hyphae are hyaline and septate but microscopy varies with species.	Identification based on microscopic evaluation of the colony.
Dimorphic agents				
Histoplasma capsulatum	Small budding yeasts within macrophages.	Colonies are slow growing, white or buff-brown in color (25^0). Yeast phase colonies (37^0) are smooth white and pasty.	Thin septate hyphae that produce tuberculate macroconidia and smooth-walled microconidia (25^0). Small, oval budding yeasts produced at 37^0C.	Demonstration of temperature-regulated dimorphism by conversion from mould to yeast phase at 37^0C. Exoantigen and DNA probe tests.
Blastomyces dermatitis	Large (8-15μm), thick-walled budding yeasts. The junction between the mother snd daughter cells is typically broad-based. Cells may appear multinucleate.	Colonies vary from membranous yeastlike colonies to cottony, white, moldlike colonies at 25^0C. When grown at 37^0C, yeast phase colonies are wrinkled, folded and glabrous.	Hyaline, septate hyphae with one-celled smooth conidia (25^0C).Large thick-walled budding yeast at 37^0C.	Demonstration of temperature-regulated dimorphism by conversion from mould to yeast phase at 37^0C. Exoantigen and DNA probe tests.
Coccidioides immitis/complex	Spherical, thick-walled spherules, 20-200 μm, mature spherules contain small, 2-5 μm endospores. Arthroconidia and hyphae may form in cavitary lesions.	Colonies initially appear moist and glabrous, rapidly, becoming downy and gray-white with a tan or brown reverse.	Hyaline hyphae with rectangular arthroconidia separated by empty disjunctor cells.	Demonstration of temperature-regulated dimorphism by conversion from mould to yeast phase at 37^0C. Exoantigen and DNA probe tests.

Fusarium spp	Hyaline, septate, dichotomously branching hyphae. Angioinvasion is common. May be indistinguishable from *Aspergillus* spp.	Colonies are purple, lavander or rose-red with rare yellow variants.	Both macro and microconidia may be present. Macroconidia are multicelled and sickle or boat shaped.	Identification based on microscopic and colonial morphology. DNA sequence based identification increasingly important.
Paecilomyces	Hyaline, septate, branching hyphae.	Colonies usually spreading broadly, white, brownish or inbright colours.	Conidiophores simple, or irregularly or verticillately branched, bearing whorls of conidiogenous cells. Conidia formed indivergent chains of various shapes.	Identification based on microscopic and colonial morphology. DNA sequence based identification increasingly important.
Dematiaceous fungi (e.g. *Alternaria, Curvularia, Cladosporium*)	Pigmented (Brown, tan or black) hyphae, 2-6 μm wide. May be branched. Often constricted at the point of septation.	Colonies are usually rapidly growing, wooly and gray, olive, black or Brown in color.	Varies considerably depending on genus and species. Hyphae pigmented. Conidia may be single or in chains, smooth or rough and dematiaceous.	Identification based on microscopic and colonial morphology.
Scedosporium spp	Hyaline, branching septate hyphae. Angioinvasion is common is *S. apiospermum*	Wooly, mouse-gray colonies. *S. prolificans* does not grow on cycloheximide-containing medium.	Single-celled conidia produced at the tips of annellides *(S. apiospermum*). Inflated conidiophores *(S. prolificans*)	Identification based on microscopic and colonial morphology.
Sporothrix schenckii	Yeast like cells of varying sizes. Some may appear elongated or “cigar shaped”. Tissue reactions forms asteroit bodies.	Colonies initially smooth, moist, and yeast-like, becoming velvety as aerial hyphae develop (25^0C).Tan to Brown pasty colonies at 37^0C.	Thin, branching septate hyphae. Conidia borne in rosette-shaped clusters at the end of the conidiophore (25^0C). Variable sized budding yeast at 37^0C.	Demonstration of thermal dimorphism. Exoantigen and DNA probe.

Penicillium spp.	Septate, branched, hyaline hyphae.	Colonies growing rapidly, powdery, effuse, green, gray, yellow or white, rarely reddish.	Conidiophores usually erect,simple or synnematous, hyaline or pale pigmented, termaly bearing one or several whorls of up wardly directed, slender metulae which bear flask-shaped to acerose phialides. Conidia produced in dry basipetal chains.	Identification based on microscopic and colonial morphology.

Table 8: Main characteristics of commonly isolated fungal species (Adapted from reference #84, 85, 86 and 88).

5. 1. 4. Susceptibility testing

Antifungal susceptibility tests will show relative activity of two or more antifungal agents against the tested organism, predict the outcome of therapy, monitor the development of resistance and investigate the therapeutic potential of newly developed agents. In vitro susceptibility of antifungal agents is standardized by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). Broth microdilution method and disk diffusion method were standardized for in vitro testing of yeasts and moulds. The reference CLSI documents include antifungal susceptibility testing of amphotericin B, flucytosine, fluconazole, ketoconazole, itraconazole, voriconazole, posaconazole and ravuconazole have published by considering data relating the minimal inhibitory concentrations (MICs). The microdilution method is based on visual reading of minimal inhibitory concentrations (MICs, μg/ml) values. Disk diffusion and E test methods are also comparable to reference methods. Following the documentation of CLSI methodology, Antifungal Susceptibility Testing (AFST) of the European Society of Clinical Microbiology and Infectious Disease (ESCMID), EUCAST developed and documented a broth dilution method. The two methods are simialar but have some modifications.[89]

Test parameter	CLSI	EUCAST
Test medium	RPMI 1640 with glutamine, without bicarbonate, glucose concentration 0.2%	RPMI 1640 with glutamine, without bicarbonate, glucose concentration 2%
Inoculum density	0.5-2.5x10^3 cfu/ml	1-5x10^5 cfu/ml
Microdilution plates	96 U-shaped wells	96 flat-buttom wells
MIC reading time point	48h	24h
MIC reading method	Visual	Spectrophotometric (530 nm)

Table 9: The major differences in test parameters of CLSI M27A3 and EUCAST broth dilution methods. (Adapted from Arikan S)[89]

Antifungal susceptibility studies frequently use systemic isolates or focus on yeasts. The ocular studies present results obtained form small sizes or focus on one particular genus or species. Latitha et al. conducted an in vitro study to investigate the activity of natamycine and voriconazole against 221 patient isolates obtained from fungal keratitis. Organisms had lower MICs to voriconaozole than natamycine. Aspergillus flavus isolates appeared least susceptible (highest MICs) to natamycine, whereas Fusarium isolates were least susceptible to voriconazole.[90]

Establishing a clinical correlation between in vitro data and clinical outcome has been difficult. Antifungal susceptibility testing can be said to predict the outcome of treatment consistent with “90-60 rule”. According to this rule, infections due to susceptible isolates respond to therapy ~90% of the time, whereas infections due to resistant isolates respond to therapy ~60% of the time. Several factors may influence the success of therapy. We need to perform antifungal susceptibility tests for the prediction of clinical outcome and optimization of antifungal therapy ocular fungal infections.

5. 2. HISTOPATHOLOGIC TECHNIQUES

5. 2. 1. Conventional microscopy

5. 2. 1. 1 Routine stains (H&E)

Hematoxylin and eosin (H&E) stain is the best stain to demonstrate host reaction in infected tissue. Stains most fungi, but small numbers of organisms may be difficult to differentiate from background. Usefull in demonstrating natural pigment in dematiaceous fungi.

5. 2. 1. 2. Special stains (GMS, Mucicarmine, PAS)

They allow for the detection of virtually all fungi. GMS stain stains hyphae and yeast forms black against a green ground. Mucicarmine stain useful for demonstrating capsular material of *C. neoformans*. Many also stain the cell walls of *B. dermatitidis*. Periodic acid-Schiff (PAS) stains both yeasts and hyphae in tissue. PAS-positive artifacts may resemble yeast cells.

5. 2. 2. Direct immunfluorescence

Immunfluorescent stain provides a fast and easy method for detecting the cell structure of fungi in clinical samples. Requires fluorescent microscope and paper filters. Background fluorescence may make examination of some specimens difficult.

5. 2. 3. In situ hybridization

In situ hybridization (ISH) most commonly used in infectious disease, including *Candida*, *Aspergillus*, *Mucor*, *Pneumocystis* and dimorphic fungi. ISH offers rapid turn around time, limited cost, and the potential for automation, together with a high degree of specificity. The sensitivity of ISH has been markedly enhanced by the use of various signal amplification methods that can detect just a few copies of target sequence. Parafin sections of ocular biopsy samples digested and fungal DNA hybridized with pan-fungal oligonucleotide probes. Visualization of labeled hybrids is applied by light microscopy.[91] Species-specific ISH probes appeared uniformly accurate, identifying correctly all organisms in which a signal was visualized by pan-fungal probes.[92]

5. 3. IMMUNOLOGIC TECHNIQUES

5. 3. 1. Cryptococcal antigen test

Ocular involvement occurs after Cryptococcal meningitis and may represent hematogenous dissemination or extention through the leptomeninges. Visual loss is the most catastrophic complication, since it is often irreversible. Eye fluid may be processed in Cryptococcal antigen test based on the direct detection of capsular polysaccharide antigen. Detection of Cryptococcal antigen is accomplished by using one of several commercially available latex agglutination or enzyme immunoassay kits.[93,94]

5. 3. 2. Antigen test for dimorphic agents

Serologic diagnosis of histoplasmosis, coccidioidomycosis, and blastomycosis employ tests for both antigen and antibody detection. Antibody detection assays include a complement fixation (CF) assay and immunodiffusion (ID) test. These tests are usually used together to maximize sensitivity and specificity, but neither is useful in the acute setting; CF and ID are often negative in immunocompromised patients with disseminated infection. Detection of fungal antigen in serum and urine by enzyme immunoassay has become very useful, particularly in diagnosingdisseminated disease. The sensitivity of antigen detection is greater in eye fluid specimens than in blood. Serial measurements of antigens may be used to assass response to therapy and for establisling relapse of the disease.[92-94]

5. 3. 3. Galactomannan test

Detection of the galactomannan polysaccharide antigen, a cell wall component of *Aspergillus*, in the serum and urine is widely used throughout the world. Platelia Aspergillus (Bio-Rad Laboratories, Marnes, France) is a sandwich ELISA kit that detects circulating galactomannan antigen using the rat monoclonal antibody. Antigen has been detected in body fluids such as BAL fluid and CSF samples. In case of endophthalmitis, aqueous and vitreous samples should be evaluated. But the volume of the fluid is not convenient for galactomannan ELISA technique, since it requires minimum 200 μl volume. Galactomannan test has not a diagnostic possibility in case of keratitis, since corneal tissue sampling is the fundemental procedure in such cases.

Sinoorbital disease or dacryocystitis and canaliculitis are the other clinical manifestations of ocular aspergillosis. Galactomannan antigen testing may be useful in those invasive infections. But, radiographic imaging of the orbit and paranasal sinuses of the soft tissue which provides details is superior than any other serologic procedures.[95-97]

5. 3. 4. Mannan test

Mannan is a cell wall surface carbohydrate that circulates during infection with *Candida* species, and the literature suggests that a positive mannan test correlates with disseminated infections. However, mannan is rapidly cleared from the blood and ocular fluid and occurs in low levels, necessitating frequent sampling for detection. A double sandwich enzyme immunoassay Platelia *Candida* Antigen (BioRad, Marnes, France) has been introduced. Serial testing seems to be necessary for accurate diagnosis.[93-94]

5. 4. BIOCHEMICAL TECHNIQUES

5. 4. 1. Metabolites (D-arabinitol)

D-arabinitol is a metabolite of certain species of *Candida*. It circulates in the serum and accumulates in the urine of patients with invasive candidiasis.[93,97]

5. 4. 2. Cell wall components (Beta –glucan)

(1→3)-β-D-glucan is a cell wall component of yeast and filamentous fungi, found to be detectable in the blood during most invasive fungal infections. The glucan assay does not detect cryptococcosis, and it is also not positive in fungal colonization.[93-97]

5. 5. MOLECULAR TECHNIQUES

Direct microscopy provides a rapid diagnosis for fungal endophthalmitis though it is less sensitive. Fungal culture is considered the “gold standard” in the diagnosis but should be carefully examined due to sapropyhtic nature of fungi. Conventional techniques help in diagnosis in up to 54 to 69 percent cases. The possible reasons for low sensitivity in conventional methods include small volume of sample, less organism load in the ocular specimen and a greater tendency of the organisms to be loculated rather than evenly distributed through the vitreous cavity. Recent developments in diagnostic molecular biology allow novel approaches in the detection of infections in

ocular fluids. Polymerase chain reaction (PCR) is used in the diagnosis of ocular fungal infections. The management of keratomycosis depends on rapid identification of the causative agent. Recent advances in molecular biology techniques have opened the door for culture-independent diagnostic methods.[98] Basic biochemistry of PCR, how its use has impacted ophthalmic practise and ways in which PCR is improving our understanding of the mechanisms of ophthalmic disease, were discussed in a detailed review of Van Gelder RN.[99] Molecular techniques are of particular use in recurrent and therapy-resistant infections. In the diagnostics of ocular mycosis molecular approaches enable the detection of fastidious microbes and of pathogens that cannot be found by culture methods. In special situations identification even to the species level is possible. In immunocompromised patients molecular techniques show more accurate results than serological ones. Therefore, PCR will be considered the gold standard to establish the etiology of infectious endophthalmitis in the near future.

5. 5. 1. PCR for direct detection of the pathogen

Molecular detection using polymerase chain reaction (PCR) for the amplification of fungal DNA from clinical samples is being applied more and more frequently for the diagnosis of ocular infections. One approach for fungal PCR has been to find species or genus specific genomic sequences, which are almost exclusively single copy genes. Another general approach has been to look for highly conserved genomic sequences that are multicopy genes in a big variety of fungi. Universal fungal primers are ideal to detect fungal infections. The target should be a multicopy gene to maximize the sensitivity of the detection method. Many different genes have been used. Ribosomal ribonucleic acid (rRNA) genes are good candidates for diagnostic PCR assays.[100] Possible targets are the 18S rRNA subunit gene, the 28S rRNA gene, and mitochondrial genes. PCR analysis of the internal transcribed spacer (ITS) regions (ITS1, 5.8S and ITS2) are used in detecting fungal species in ocular samples. Using a panfungal PCR assay may allow the detection of a wide variety of different fungi.[101,102] Sensitive and specific PCR assays to detect fungal DNA are an important part of diagnostic approach. A good DNA extraction methods is essential before the amplification of DNA. As

mainly in house PCR assays are performed, standardization is strongly needed.The assay would then be useful as a single screening tool for the detection of all fungal infections. This is particularly important, as serial monitoring will likely be required, and using one assay rather than a battery of assays would help keep the amount of sample needed and cost of testing down. Detection limits would be at least as low as 1cfu/ml and finally a species-specific step should be added to identify the amplified fungal DNA. Amount of required sample is particularly important for ocular infections, since sampling from eye may not provide adequate material.[102]

Molecular diagnosis tool has not been widely available for use in clinical laboratories. There is a urgent need to develop a diagnostic system that could be used to examine bacteria, fungi, parasites and viruses in very small samples. Sugita et al. proposed an algorithm for molecular screening of ocular samples. DNA was extracted from the aqueous humor or vitreus fluids. Two steps of PCR were performed as step 1 for viruses and toxoplasma, in the same run, using multiplex targets, step 2 for bacterial and fungal rDNA detection. These results indicated that based on the confidence of the diagnosis, PCR system could be used to design appropriate early treatments for ocular diseases.[103]

The microbiological study identifies the fungal spectrum in acute or after surgery, delayed-onset, or chronic endophthalmitis. DNA amplification of panfungal - sequences in DNA extracted from ocular samples is a new tool for the etiological diagnosis of endophthalmitis. The most successful way to identify fungi in endophthalmitis is the association of conventional cultures and panfungal PCR on vitreous samples. Both techniques should modify our future therapeutic strategies. Diagnosis of fungal keratitis begins with clinical suspicion, and either culture or corneal biopsy confirms it. Although many characteristic morphologic features have been attached to fungal ulcer, none is pathognomonic. The Standard techniques for culture in fungal infections are complicated by many factors. Major limitation is the time factor, because fungi are often slow growing. PCR assays produce results in 8 hours, whereas culture confirmation take almost 10 days. Another limitation is the poor sensitivity rate of culture which is known to vary widely from place to place. Ocular cultures are positive

in 36-73 of case.[104] Most clinicians and microbiologists thus resort to direct microscopic examination of wet mount prepared from corneal scraping for a rapid diagnosis. But, KOH wet mount and Gram's smear have inadequate rates for the diagnosis of fungal infections. More over, identification on species or genus level can not be possible in most cases. PCR has reportedly been found to be of paramount value to the ophthalmologist, not only for the diagnosis of fungal keratitis but also delayed onset endophthalmitis, among others.[105,106] PCR based test can detect both viable and nonviable organisms. Although various advantages have been attributed to PCR, the technique has various limitations. Some of them are logistic but some of them technical. There is an urgent need of optimization and standardization since most of them are still in house protocols. PCR appears to be promising as a means to diagnose fungal infections, offering some advantages over culture methods, including rapid analysis and the availability to analyse specimens far from where collected, however the possibility of false-positive results needs to be always considered.

PCR may be useful when added to the protocol of management of cases of pediatric fungal endophthalmitis.[107] Use of PCR increases the laboratory rate of identifying the pathogen by 20 %, confirming the technique is very useful for the endophthalmitis specimen.[108,109]

To evaluate the utility of PCR on intraocular clinical specimens aqueous humor and vitreous fluid were analysed as an etiologic diagnostic tools relative to microbiological culture methods in infectious endophthalmitis. Conventional bacterial and mycologic cultures and PCR for eubacterial and panfungal genomes were applied for etiologic diagnosis on pairs of aqueous humor and vitreous fluid obtained from 72 patients with clinically established infectious endophthalmitis. Fungal and bacterial colonies were recovered in 27 (37.5 %) of 72 patients. PCR were found to be positive in all 72 patients. PCR significantly increased the clinical sensitivity over culture by 62.5 %.[110]

The sequencing of PCR products of the 16S rRNA gene for bacterial identification or 5.8S rRNA gene for fungal identification have been utilized by various authors The utilization of molecular methods has been explored in ophthalmology field, especially

for the diagnosis of endophthalmitis, because they represent a diagnostic approach with a marked increase in positivity in relation to conventional methods. Due to the small quantity of specimen collected, and consequently less quantity of microorganisms detected in the aqueous and vitreous humors, the nested PCR technique is indicated for the diagnosis of endophthalmitis, by increasing substantially the sensitivity of DNA detection. The amount of microbial DNA that can be detected by nested PCR can be as low as 1 fg. Therefore, the real-time PCR technology could be a potential technique for use in ophthalmology. Real-time PCR combines amplification and detection of a DNA sequence target by detection using specific fluorochrome- labeled probes, or based on the determination of denaturation temperature of a double-stranded DNA sequence ("melting temperature" - *Tm*) labeled with an intercalating fluorescent substance. Moreover, due to the possibility of quantifying DNA present in the specimen, its application can contribute to the differentiation between true infection and a possible contamination of the anterior chamber by microorganisms present in the conjunctival flora in patients recently submitted to intraocular surgery.[106,108,110]

5. 5. 2. Molecular methods for the identification of fungi

Numerous molecular techniques including PCR-based technology and microarray technology have been used for the identification of fungal isolate. Genotypic identification of fungal species from ocular sources is performed by using DNA sequencing and phylogenetic analysis. DNA is extracted, purified and ITS region is amplified and sequenced.[111] Genus and species-specific identification of fungi using conventional techniques generally require 3-7 days. In contrast, the use of PCR and sequence analysis is capable of identification in less than 24 hours.[112] Analysis of sequences (5.8S/ITS region) from the database confirmed that DNA sequencing can be used to differentiate fungi at the species level.[113] Other molecular methods used for fungal identification are restriction fragment length polymorphism analysis of ITS region, hybridization of specific probe, and the specific PCR.

Species-specific probes might be used for the identification of the most important species of corneal pathogenic fungi. However the range of fungi causing keratitis is

significantly wide. Therefore some species causing infection could remain unidendified by these molecular methods. The sequencing of ITS region allows this requirement. Small size of the DNA fragment permits its sequencing in both directions at once, and the obtained sequence gives enough information to identify the fungal species.[113] Specific DNA microarray combining multiplex PCR and consecutive DNA chip hybridization to detect fungal genomic DNA in clinical samples other than ocular ones, was evaluated.[114] This method can also be performed for ocular samples.

Species-specific identification of a wide range of fungal pathogens can be performed by Luminex xMAP hybridization technology. This method is a kind of hybridization assay, which permits the analysis of up to 100 different target sequences in a single reaction vessel.[115]

Rapid detection of fungal keratitis with DNA-stabilizing FTA filter paper is a promising method, published recently. Specimens were collected from ocular surfaces with FTA filter discs. Collected cells are lysed and DNA is stabilized on the paper. Filter disc were directly used in PCR reactions to detect fungal DNA. Clinical specificity was 91.7 % to 99.0 % and the method was rapid and inexpensive.[116]

5. 5. 3. Strain typing with molecular methods

Additional molecular epidemiologic data is required when more than one isolate is recovered from the same clinical setting, or when unusual isolates are recovered multiple times within the same institution. DNA fingerprinting techniques are restriction fragment length polymorphism, with or without hybridization probes, sequencing, random amplification of polymorphic DNA (RAPD), pulsed field gel electrophoresis, and other electrophoretic karyotyping methods. RAPD technique was used for clustering of *Aspergillus ustus* eye infections in a tertiary care hospital. The seven ophthalmologic strains were genetically identical by the RAPD method, indicating a possible common source.[117]

Genotyping studies have shown that DNA sequence based methods are useful for species identification and subtyping of fungal isolates.[118,119] Molecular techniques are also useful for the management of outbreaks of ocular infections.[120] PCR with short,

nonspecific primers is an inexpensive, fast, reproducible and discriminatory DNA typing tool for effective epidemiologic surveillance of clinical and enviromental isolates of fungi. Airborne exposure in the operating rooms has been described, including clusters of *Acremonium* endophthalmitis associated with a contaminated ventilation system, ocular aspergillosis associated with a hospital construction.

Molecular methods are highly sensitive and specific for the detection, identification and typing of fungal agents in patients with ocular infections.

5.6. Other diagnostic methods

In vivo confocal microscopy enables to understand the ocular pathology at a cellular level.[121] The early detection of fungal structure on confocal microscopy with no growth on culture alters the management of the disease. Confocal microscopy is a relatively new, noninvasive technique for imaging the cornea in normal and diseased states.[122] Avunduk et al found that confocal microscopy in experimentaly induced *A. fumigatus* keratitis in rabbits was more sensitive than culture on days 14 and 22 in treated and untreated control rabbits.[123]

6. EXPERIMENTAL MODELS

6. 1. IN VIVO MODELS

As ocular fungal infections are relatively rare compared to the infection of bacterial agents, it is important to use the proper kind of animal and the most effective method in order to draw correct conclusions. An appropriate animal model is crucial for prospective studies designed to identify and evaluate risk factors affecting the development of fungal infections. Investigation of ocular mycosis requires animal models that allow high reproducibility and sensitive quantitation. For example, an animal model would permit the evaluation of the roles of ocular trauma, coinfection with bacteria, and contact lenses in the development keratitis. Moreover, an animal model also would facilitate investigations exploring the pathophysiology, cell biology, genetics, immunology, and therapy of this disease. A prospective animal model must satisfy several basic criteriafor serious consideration for long-term studies. The most fundamental criterion is that the disease must conform to Koch's postulates. Disease

should be produced by live, infectious organisms. Viable colonies must be isolated from the diseased tissues and grown in pure culture. In addition, the model should display clinical features comparable to the human counterpart. The efficacy of different treatment strategies has been studied extensively but the pathogenesis of ocular infections remain unknown mechanisms. Pigs, rabbits, hamsters are used for experimental ocular infections. Rats and mice are the most widely used animals, since they are easy and cheap to keep in large numbers and there are rich resources of molecular reagents such as antibodies for these animals. Not only the host factors, and defence mechanisms, but also the virulence factors of fungi were evaluated in experimental models of ocular mycosis. The experimental protocols should be approved by "Association for Research in Vision and Ophthalmology" and local ethics committees.[124-126]

6. 1. 1. Mouse models

Outbred NIH Swiss and inbred BALB/C mice widely used as target animals. Depending on the fungal species, immunosuppression is frequently necessary, for this purpose methylprednisolone (100mg/kg) or cyclophosphamide are used. Immunosupression increases susceptibility to corneal mycosis. Animals are pretreated with intraperitoneal cyclophosphamide 180 mg/kg at 5,3 and 1 days before corneal inoculation.[127] Under ketamine (37.5mg/kg) and xylazine (1.9mg/kg) anasthesia, superficial wound is generated on corneal surface of eye for keratitis model. The mice is killed and the eyes enucleated after hours or days, depending on the hyphothesis of the study. Histologic examination, quantitative microbial culturing and molecular analysis can be performed.[128]

Developing a mice model provide valuable knowledge for the understanding of immune response of the host and the pathogenesis of ocular infection. Successful corneal surface inoculation would enable pathogenic studies of microbial adherence and the early events of fungal keratitis. A mouse model would offer oppurtunities for studies of immunology and molecular genetics of oculomycotic pathogenesis.[129]

Injuring the corneal epithelium by scarification is a standard method for eliminating

corneal barriers. Fungal inoculum is $1x10^3$ CFU to lead keratitis. Cyclophosphamide is a well known immunosuppressive agent which allows prolonged fungal persistance in mouse cornea.[126]

In another model of fungal keratitis central corneal epithelium is removed in a diameter of 2 mm before a full thickness rat corneal button was placed on the mouse cornea. Fungal inoculum is injected into the space between the two corneas. The eyelid is sutured to secure the rat corneal button, and another inoculum is injected into the conjunctival sac. In this model expression of inflamatory cytokines such as MIP2, KC, IL-1 and IL-6 were determined using ELISA and RT-PCR assays.[130]

A murine model of contact lens-associated *Fusarium* keratitis was established. *Fusarium* grown as a biofilm on contact lenses induced keratitis on injured corneas. Findings demonstrated that the ability to

form biofilms (comparing ATCC with clinical isolates) is a key determinant of *Fusarium* pathogenesis in vivo, that *F. solani* is more virulent than *F. oxysporum* regardless of biofilm thickness, and that the ability to form biofilms may contribute to survival of both species.[131]

6. 1. 2. Rabbit models

New Zealand albino rabbits were most frequently used model animals for ocular infections. Treatment modalities were mostly studied in rabbits. Intravitreal and intravenous injections are easier in rabbit models than those in small animals.[132,133]

Contact lens induced keratitis model was established in rabbits. Intramuscular ketamine (35mg/kg) and xylazine (5mg/kg) induced anesthezia in experimental animals. Corneal anesthezia is performed with topical preperations such as 0.2 % novocaine. Corneal epithelium can be removed and fungal inoculum can be transferred to the denuded cornea with a large-bore pipette tip and covered with a contact lens. The lids can be closed with sutures to prevent contact lens extrusion.[134]

In another rabbit model, results of intrastromal amphotericin B treatment was evaluated. AmB at concentrations of 5 and 10 μg per 0.1 ml did not induce obvious toxicity. However, when the concentration increased to 20 μg per 0.1 ml or more, corneal edema,

corneal epithelial erosion and severe neovascularization appeared. A single intrastromal injection of 10 μg AmB achieved an effective drug level in corneas which was maintained for up to 7 days as an adjunctive treatment for deep recalcitrant fungal keratitis.[135]

Clinical scoring system is used to grade conjunctival hyperemia, corneal clouding, diameter of corneal neovascularisation and hypopyon level in experimental models.[136]

Fungal endophthalmitis can be created in immuno competent and immunosuppressed rabbits.[137]

Different antifungal treatment regimens for experimental *Candida* endophthalmitis models were evaluated in our study, as well as in another experimental study.[41, 138]

6. 1. 3. Rat models

Rats are superior models not only for their suitable size and immune response, but also because the size of their eyes makes controlled surgical procedures easier.[139] Wistar rats or Lewis rats are used as experimental animal for keratitis model. All corneas are examined before inoculation. The procedure is performed under an operation microscope. Initially, a half thickness linear blade incision is made 2 mm from the center of cornea. Microliter syringe and 30 G needle is used for the incision. Fungal inoculum is injected into the stroma.

6. 1. 4. Other models

The large size of the pig eye, its anatomical similarity to the human eye, and the ease in fitting soft contact lenses to the pig eye made this a promising host for development as an animal model contact lens associated keratitis. New treatment options or new applications of a known drugs were assayed in pig models. Novel surfactant-based elastic vesicular system for ocular delivery of fluconazole were evaluated using porcine cornea.[140] Primate model was found valuable as an experimental model for ocular fungal infections.[141]

6.2. IN VITRO MODELS

In vitro models have greatly pathogenicity in its single consecutive steps at a cellular level advanced our knowledge about fungal virulence by providing a means of understanding the process of mycotic infections. Corneal epithelial cells could be isolated and inoculated by *Aspergillus fumigatus* in an in vitro cell culture model.[142]

Endogenous *Candida* endophthalmitis was simulated in a cell-culture model. Endothelial toxicity of caspofungin was evaluated in cultured human corneas. Possible toxic effects of caspofungin in corneal endothelial cells (CEC), primary human trabecular meshwork cells (TMC) and primary human retinal pigment epithelium (RPE) cells were evaluated after 24 h. No corneal endothelial toxicity could be detected after 30 days of treatment with 75 μg ml (-1) of caspofungin.[143]

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