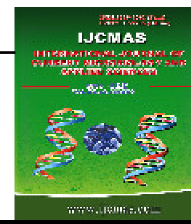

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 6 (2013) pp. 307-314**
http://www.ijcmas.com

Review Article

A review of fungal keratitis: etiology and laboratory diagnosis

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A B S T R A C T

Fungal keratitis is an inflammation of the cornea caused by fungi. This infection is difficult to treat and it can lead to severe visual impairment or blindness. It is worldwide in distribution, but is more common in the tropics and subtropical regions. Trauma is the major predisposing factor, followed by ocular and systemic defects, prior application of corticosteroids, and prolonged use of antibiotic eye-drops. Fungal keratitis can be caused by Moulds, Yeasts, Dimorphic fungi. 105 species of fungi have been identified to cause keratitis but Fusarium species and Aspergillus species are responsible for 70 % of cases. The diagnosis of fungal keratitis is usually difficult. The clinical suspicion by ophthalmologist is unequivocally, key element in making diagnosis of fungal infection of cornea. A wide range of conventional and molecular techniques are currently available for laboratory diagnosis of fungal keratitis. Early diagnosis and appropriate treatment are essential to avoid blindness.

Keywords

Keratitis; Diagnosis; *Fusarium; Aspergillus;* Dimorphic fungi.

Introduction

Keratitis is an inflammation of the cornea (the clear, front part of the eye) and is often caused by Bacteria, Viruses, Amoeba, Fungi (Centers for Disease Control and Prevention, 2013). There are two types of keratitis 1.Infectious ulcerative keratitis (IUK), caused by infectious pathogens 2. Noninfectious ulcerative keratitis (NIUK), which has a clinical presentation similar to that of IUK but no known infectious cause and is a diverse disease associated with systemic disorders (Kuo *et al.,* 2012). Fungal keratitis is an inflammation of the cornea caused by a fungus.

Fungal keratitis was first described by Leber in 1879 (Centers for Disease Control and Prevention, 2013; Singh,2011).

According to the World Health Organization report, it is estimated that ocular trauma and corneal ulceration result in 1.5 to 2 million new patients of corneal blindness annually, posing a major public health problem for developing countries (Saha *et al.,* 2009).

Fungi cannot penetrate the intact corneal epithelium and do not enter the cornea

from episcleral limbal vessels. The principal routes of inoculation are introduction concurrent with a penetrating or perforating wound, either mechanical injury or surgery, and introduction through an epithelial defect (Jones, 2006).

In tropical climates as in South Florida, Bangladesh, South India and Nepal, fungal keratitis is reported to be from 17-40%. In temperate climates such as Britain and Northern United States, incidence of fungal keratitis is very low (Bharathi *et al*., 2003)

Etiology

A total 105 species of fungi, classified in 56 genera, have been identified as the etiological agents of fungal keratitis (Tilak *et al*., 2010) but *Fusarium* species and *Aspergillus* species are responsible for 70 % of cases (Bharathi *et al*., 2003). On morphological basis, causative fungi can be classified as Filamentous, Yeast and Dimorphic form (Wiggs *et al*., 2009).

Filamentous fungi

These are multicellular, elongated, branching structure called as hyphae and reproduce by spore formation which can be unicellular (microconidia) or multicellular (macroconidia). Hyphae are of two types- septate (having cross walls called as septa) and non septate. Filamentous fungi are also divided into two groups (Mcginnis). Moniliaceae: Hyaline hyphae and conidia; Dematiaceae: Dark hyphae and conidia (Table 1)

Yeasts

These are single cells and reproduce by budding. They may form the chains of elongated cells called as pseudohyphae (Table 2)

Dimorphic fungi

These are found in two forms- Filamentous form (saprophytic phase): At 22º C or room temperature; Yeast form (parasitic phase): At 37º C or body temperature (Table 2).

Laboratory diagnosis
Sample Collection

Corneal scraping is the ideal sample in keratitis. Sample is collected after anaesthetising the cornea by proparacaine hydrochloride (0.5%). With the help of sterile Kimura spatula or Bard-Parker blade No.15 or Iris repositor, scraping is done by applying multiple, moderately firm, unidirectional strokes, under slit lamp illumination. Material is collected both from the base as well as from the edge of the ulcer (Nayak, 2008).

Microscopy

Sample is spread onto labelled slides in a thin, even manner for 10% KOH wet mount & Gram's staining (Geethakumari *et al*., 2011). Then examine the slide under microscope for fungal hyphae (Septate and non septate), yeast and pseudohyphae.

The preferred stains for identification of hyphal fragments and yeasts in smears of corneal scrapings are acridine orange and calcofluor white. Each of these techniques requires use of a fluorescent microscope. The optical activity of acridine orange is due to the binding of the dye to deoxyribonucleic acid (DNA) of bacteria, fungi, and amoebae to form a green-fluorescing complex. Acridine orange is more sensitive than the Gram stain in detecting microorganisms in clinical specimens and is, therefore, a rapid and sensitive method for screening all corneal

Table.1 Filamentous fungi causing Keratitis

Septate				**Non septate**
Moniliaceae (non pigmented)			**Dematiaceae (Pigmented)**	
Acremonium	Exserohilum	Periconia	Alternaria	Absidia
Acrostalagmus	Fonsecaea	Phaeoisaria	Aureobasidium	Mucor
Arthrobotrys	Fusarium	Phoma	Bipolaris	Rhizopus
Arthrographis	Fusidium	Pithomyces	Curvularia	
Aspergillus	Geotrichum	Rhizoctonia	Exserohilum	
Aureobasidium	Glenospora	Scedosporium	Lasiodiplodia	
Beauvaria	Graphium	Scopulariopsis	Phialophora	
Botryodiplodia	Helminthosporium	Stachybotrys	Torula	
Botrytis	Hormodendrum	Syncephalastrum		
Calcarisporium	Metarhizium	Tetraploa		
Cladosporium	Microsphaeropsis	Trichoderma		
Colletotrichum	Microsporum	Trichophyton		
Cylindrocarpon	Nigrospora	Trichosporon		
Drechslera	Paecilomyces	Verticillium		
Epidermophyton	Penicillium	Volutella		

Table.2 Yeasts & Dimorphic fungi causing Keratitis

Yeasts			**Dimorphic fungi**
Candida	Pichia	Sporotrichum	Blastomyces
Cryptococcus	Rhodosporidium	Torulopsis	Sporothrix
Monilia	Rhodotorula	Trichosporon	Paracoccidioides
Oospora	Saccharomyces	Ustilago	Histoplasma*

(*) Histoplasma is rare cause of keratitis.

Figure.1 (A) Calcoflour white staining (B) Acridine orange staining.

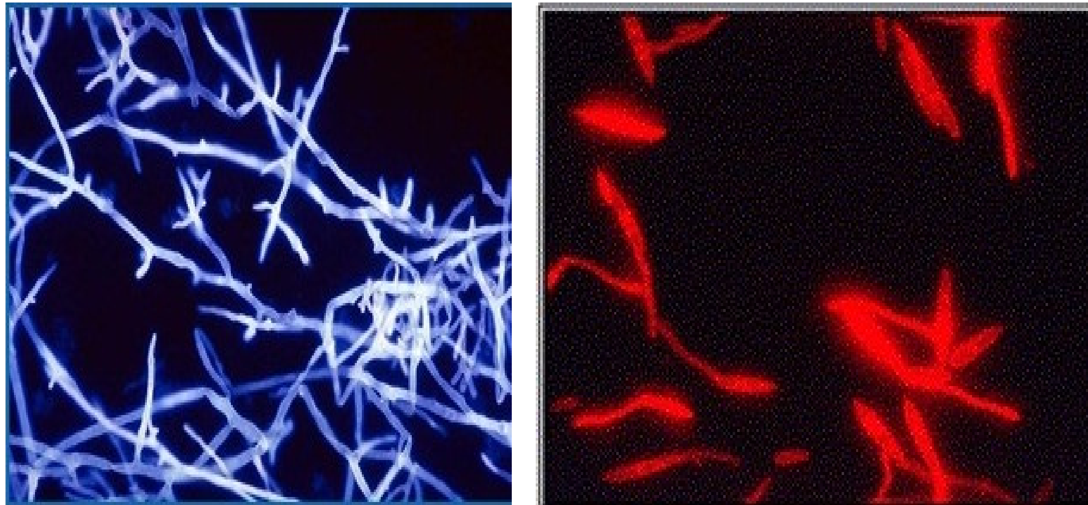
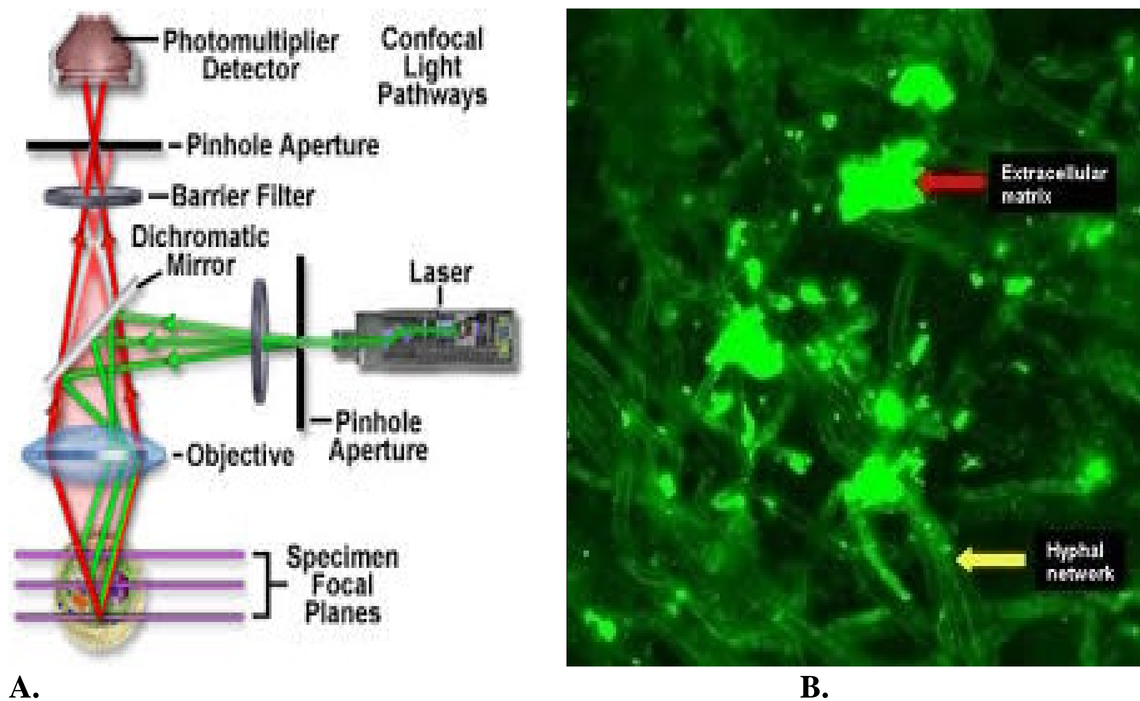


Figure.2 (A) Diagrammatical working of Confocal microscope (B) Image of Aspergillus species under Confocal microscope.



A. **B.**

smears in suspected infectious keratitis. If microorganisms are detected by acridine orange stain, the slide can be washed and stained with Gram or other specific stains (Jones, 2006).

Confocal Microscopy

It is a non-invasive fluorescent imaging technique that uses lasers of various colours to scan across a specimen with the aid of scanning mirrors. The point of illumination is brought to focus in the specimen by the objective lens. The scanning process uses a device that is under computer control. The sequences of points of light from the specimen are detected by a photomultiplier tube through a pinhole. The output is built into an image and transferred onto a digital computer screen for further analysis. The technique employs optical sectioning to take serial slices of the image. The slices are then stacked (Z-stack) to reconstruct the three-dimensional image of the biological sample. Optical sectioning is useful in determining cellular localization of targets. The biological sample to be studied is stained with antibodies chemically bound to fluorescent dyes similar to the method employed in fluorescence microscopy. Unlike in conventional fluorescence microscopy where the fluorescence is emitted along the entire illuminated cone creating a hazy image, in confocal microscopy the pinhole is added to allow passing of light that comes from a specific focal point on the sample (Wikipedia The Free Encyclopedia,2013).

Culture

Sample is inoculated on the surface of culture media like sheep blood agar, chocolate agar, McConkey agar and Sabouraud Dextrose Agar (SDA) in a raw of ‘c’ shaped streaks. SDA plates is incubated at 27°C and examined daily and discarded if no growth is seen after 21 days. The remainders are incubated aerobically at 37°C and evaluate at 24 hours and 48 hours. Discard after 72 hours if no growth (Bharathi *et al*., 2003).

Identification

Culture Characteristics

The major macroscopic features remarkable in species identification are the growth rate, colour of the colony (on obverse and reverse side), and thermo tolerance.

Microscopic appearance

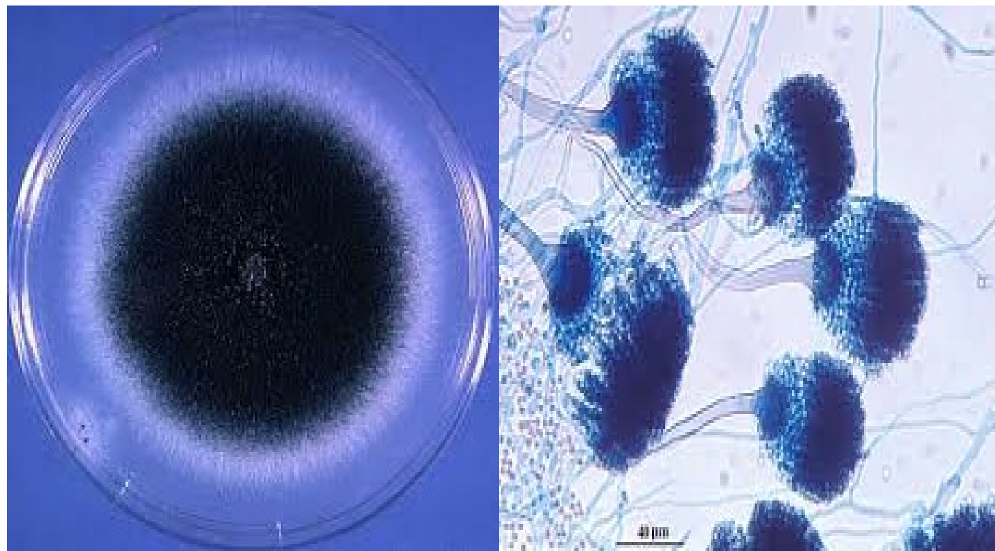
The basic microscopic morphology is different for different species. Yeast isolates were identified by direct microscopy with Gram’s stain, germ tube formation in serum, and negative staining. Identification of filamentous fungi are based on microscopic appearance on slide cultures stained with lactophenol cotton blue (LPCB) and included septate and branching hyphae, color, size, shape, texture, and formation of conidia (Chandra, 2009; Kindo *et al*., 2009).

Molecular Technique

Molecular diagnosis of ocular infections is based on DNA detection of microorganisms by polymerase chain reaction (PCR) in ocular samples (Ferrer and Alio, 2011). PCR is the enzymatic exponential amplification of a specific target region using short primers, leading to detectable amounts of amplified DNA from one or a few original sequences. A less time consuming process involves the

Figure 3 'C' shaped streak on Sabouraud Dextrose agar plate.

Figure.4 (A) Growth of *Aspergillus niger* on culture media (B) *Aspergillus niger* in LPCB mount**.**



A. **B.**

use of real-time PCR. During real-time PCR, the accumulation of PCR products is measured automatically during each cycle in a closed tube format using an integrated cycler/fluorimeter (Atkins and Clark, 2004). Internal transcribed spacer region (ITS) that contained the target gene (5.8S rRNA gene) is amplified by Polymerase chain reaction (PCR) and seminested PCR to detect fungal DNA (Kuo *et al*., 2012).

Serological tests

There are no specific immunological test for confirmation of fungal keratitis. Moreover, diagnosis is finally based on demonstration of organism in direct smear and culture confirmation, which is sufficient to the extent that serological tests are not required (Chandra, 2009).

In conclusion, the key element in the diagnosis of mycotic keratitis is the clinical suspicion by ophthalmologists. The regional information of etiological agent is very important as this will help to have a high degree of clinical suspicion in starting the appropriate initial treatment before getting the microbiological confirmation. Conventional as well as molecular techniques are needed for early and reliable diagnosis.

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