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RESEARCH ARTICLE

DIAGNOSIS OF KERATOMYCOSIS: AN UPDATE

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ABSTRACT

Keratomycosis is defined as infiltration of the cornea by fungi. Majority of cases are infiltrated by filamentous fungi. Keratomycosis is an ophthalmic emergency as prompt and reliable diagnosis with efficient treatment can prevent vision loss. Improper diagnosis with injudicious use of medical therapy may result in worsening of the condition with poor clinical outcome. Traditional diagnostic methods like staining and culture are highly specific but with low sensitivity. Newer diagnostic methods like Confocal Microscopy, Fluorescent Microscopy and PCR have greater sensitivity as compared to traditional methods. With better diagnostic tools a good clinical outcome can be ensured, thus the reducing ocular morbidity especially in tropical countries like India. Prompt and reliable diagnosis is the main-key to reduce the ocular morbidity in infectious keratitis.

INTRODUCTION

Keratomycosis is synonymous to fungal corneal ulcer which is characterized by stromal infiltration caused by fungi. Fungi are ubiquitous organisms as they are present in environment as a saprotrophs. These fungi cause infection when there is a breach in corneal epithelium as epithelium acts as a barrier to microorganisms, but some of them especially *Fusarium* spp. may penetrate the intact cornea. World Health Organization reported that corneal diseases are the major cause of monocular blindness after cataract. Around 1.5 to 2 million new cases of corneal ulcer are reported annually (Whitcher *et al*., 2001).

Epidemiology

Keratomycosisis a major cause of mono-ocular visual disability in developing countries (Miño de Kaspar *et al*., 1991). The first case of keratomycosis was reported by Professor Theodor Leber in 1879 and since then there has been a rapid rise in the diagnosis of fungal corneal ulcer (Kaufman *et al*., 1965; Anderson *et al*., 1963). This is either due to increased awareness among clinicians with better diagnostic facilities or really increasing number of infected cases. Injudicious use of corticosteroid and antibiotics increases the frequency of fungal corneal ulcer. It is estimated that in India, prevalence of fungal corneal ulcer is 44-47% (Srinivasan *et al*., 1997; Polack *et al*., 1981; Thomas, 1994) compared to 17-36% worldwide (Upadhyay *et al*., 1991; Rosa *et al*., 1994; Liesegang and Forster 1980). High prevalence of fungal corneal ulcer is attributed to tropical climate and agriculture as a major occupation. Indians are at a high risk of keratomycosis due to high vulnerability for occupational trauma especially by organic materials and sand particles.

Risk factor: Fungi are opportunistic pathogens as they cause tissue invasion only in immuno-compromised state or after direct entry of pathogens in a closed ocular space. Trauma especially by vegetative material, sand or dust particles and the tail of an animal is the major predisposing factor in 55-65% cases of fungal corneal ulcer (Srinivasan *et al*., 1997; Upadhyay *et al*., 1991; Bharathi *et al*., 2003; Panda *et al*., 1997). Despite reducing inflammation, topical corticosteroid instillation enhances fungal growth (Mitsui and Hanabusa 1955; Agarwal *et al*., 1963; Forster and Rebell, 1975). Contact lens users are also at high risk for developing ulcer especially by *Fusarium* spp. as there are micro epithelial erosions with the use of contact lens (Höflin-Lima AL, Roizenblatt, 2002; Choi *et al*., 2001; Alfonso *et al*., 2006; Khor *et al*., 2006; Nelson *et al*., 1994). Other risk factors include corneal surface disorders like dry eyes, corneal dystrophy, exposure keratitis and previous corneal surgery (Anderson *et al*., 1959; Cohen *et al*., 2000; Thomas, 2003).

Etiological agents: Filamentous fungi are the major pathogen causing fungal keratitis. Hyaline fungi (*Aspergillus* and *Fusarium* spp.) have a major preponderance followed by

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Phaeoid fungi (*Curvularia* and *Bipolaris* spp.) (Vijaya *et al*., 2001; Basak *et al*., 2005). *Candida* spp., a yeast like fungi, have a low preponderance in developing countries. Until recently more than 70 different species of fungi have been identified as the causative agent for fungal corneal ulcer (Prajna *et al*., 2002).

Diagnosis: Early and prompt diagnosis of fungal corneal ulcer is essential to prevent devastating complications. A good clinical evaluation including relevant clinical history and slit-lamp examination is required to suspect a case of fungal corneal ulcer. The duration of infection in fungal ulcer is usually long. Fungal keratitis can involve any part of cornea. In untreated cases, fungal corneal ulcer is seen to have feathery borders or hyphate edges. Entire lesion or large area of the lesion is elevated well above the surrounding area. Ulcer may be surrounded by a clear rim (devoid of fungi) known as 'Immune Ring of Wessley'. This ring represents immune response against fungi. Non-specific satellite lesions or discrete stromal infiltrates surrounding the ulcer is another common finding. Fungal keratitis, caused by phaeoid fungi has brown–black pigmentation on the surface of corneal ulcer. Hypopyon, the collection of exudates in anterior chamber, is present in around 50% of cases. Signs of inflammation like pain and ciliary congestion are usually minimal compared to bacterial keratitis. Typical clinical features of fungal corneal ulcer may be masked by instillation of drugs.

Laboratory diagnosis: Laboratory diagnosis is essential for accurate diagnosis of etiological agents. Prompt and effective treatment, not only slows the progression of the disease, but also results in early healing of the ulcer. False negative diagnosis not only delays the specific antifungal therapy, but also injudicious use of medication leads to rapid growth of organisms. For a better and reliable diagnosis, cornea is scraped several times so that adequate sample material is obtained for microbiological examination. Fungal keratitis is diagnosed by the following modalities:

1.Direct microscopic examination
2.Culture
3.Corneal biopsy
4.Newer diagnostic tools

Sample collection: Material for direct microscopic examination, culture and other diagnostic methods should be collected from central deeper area of lesion. A failure to collect adequate sample material from the relevant site is the major cause for false negative results. Sample is collected using a sterile 15 no. surgical Bard Parker blade after instillation of 2% lignocaine hydrochloride. Debris and necrotic material is removed and discarded. Calcium alginate swab moistened with trypticase soy broth or a rayon swab moistened with thioglycolate broth have better results (Jacob *et al*., 1995). In deep ulcer, scrapings should be done from the margins so as to avoid chances of perforation.

Direct microscopic examination: These are rapid methods that detect fungal elements in corneal scrapes, but require considerable expertise. Following techniques are commonly used:

Gram's stain: It is a commonly performed test used for microbiological evaluation. This stains the fungal cytoplasm, seen as violet colored, resulting in gram positive fungal hyphae (Fig. A). Yeast cells like *Candida* are seen as a budding yeast cell. Apart from fungal element, this helps in identification of concomitant bacterial infection. Sensitivity of Gram's stain to fungi is poor being 55 to 88% (Panda *et al*., 1997). Proteinaceous material and thick smear interfere in the identification of fungal elements. Sometimes stain particles mimic as a fungal elements. Pus cells also interfere with yeast cell identification.

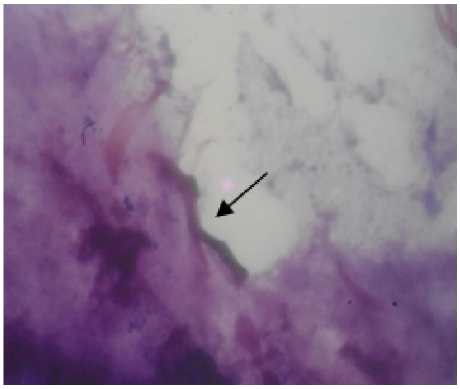

Fig. A. Fungal elements on Gram's stain (1000 x) [arrowed]

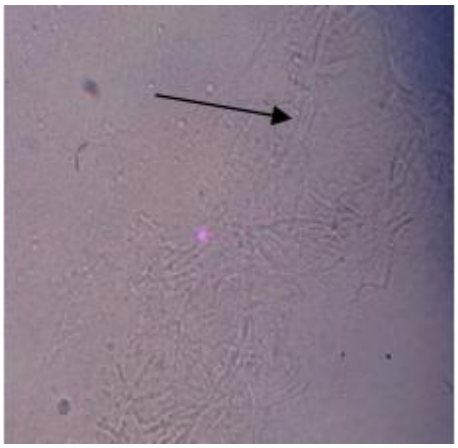

Fig. B. Hyaline fungal hyphae on 10% KOH wet mount (400 x) [arrowed]

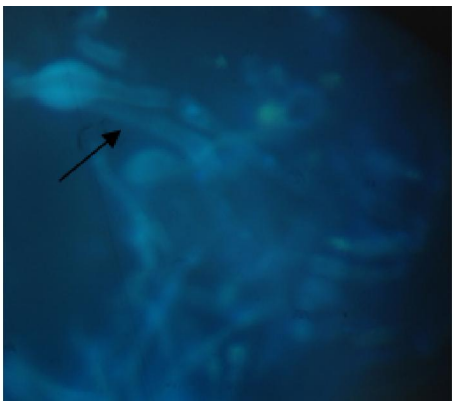

Fig. C. Fungal elements on Calcoflour white stain mixed with KOH (400 x) [arrowed]

KOH wet mount: 10% Potassium hydroxide (KOH) wet mount is the oldest and widely used method for detection of

fungal element in corneal scrape. Instead of KOH, NaOH can also be used. Potassium hydroxide partially digests the protein debris, host cells and stromal collagen, leaving the fungal cell wall intact. 10% Potassium hydroxide solution is prepared by dissolving 10gram of KOH into 90 ml of distilled water. 10ml glycerol is added in 10% KOH to prevent drying. Classically, a few drops of 10% KOH are put over corneal scrapes. If scrape is thick then it can be spread with the help of sterile slide or 15 no. blade. Cover slip is placed over the wet mount and left at room temperature for 5-10 minutes. The slide is then examined initially at 100x magnification and later on higher magnification at 400x. Complete dissolution of host cell is required for the detection of fungal elements. These elements including mycelia or yeast cells are seen as refractile structures (Fig:B). They are further categorized as hyaline or phaeoid, septate or aseptate and branched or non-branched hyphae. Visibility of hyphae is increased after by increasing the time duration.

Instead of using plain 10% KOH wet mount, some counter stain substances like India ink, lactophenol cotton blue, ethylene blue and calcofluor white stain can also be used. These substances bind with fungal cell wall component and gives counter stain to the fungal elements resulting in better identification. But they failed to differentiate the types of fungi whether they are hyaline or phaeoid. KOH wet mount is easy, simple to perform, less time consuming and cost effective. It can also be performed in remote areas, where other facilities of diagnosis are not available. But certain expertise is required to identify and interpret the results. Sensitivity of KOH varies from 33 to 100% (Liesegang and Forster, 1980; Sharma *et al*., 1998). Lisegang *et al*., found 33% sensitivity of 10% KOH, compared to 100% sensitivity as reported by S. Sharma in detection of fungal elements in culture proven cases. Sensitivity of KOH is increased after addition of counterstaining substance like lactophenol cotton blue or fluorescent calcofluor white stain.

Giemsa stain: Giemsa is a compound stain formed by interaction of methylene blue and eosin. When methylene blue is exposed to acid, alkali or ultraviolet rays, a large number of oxidation products (methylene azure) are formed. In this staining, smear is fixed in methanol and then exposed to Giemsa stain. This stain is used to evaluate the cytology of corneal scrapes, aqueous and vitreous aspirates, pus and necrotic materials. It stains yeast cells and fungal hyphae as purplish blue. Apart from fungi, *Acanthamoeba* cysts are easily visualized. Sensitivity of Giemsa stain varies from 66-85% in culture proven fungal ulcer cases (Gopinathan *et al*., 2002). Drawbacks of this stain are similar to the Gram's stain like artefact, debris and thick smear that interfere with the results.

Gomori-Methenamine Silver Stain (GMS): It is one of the most selective methods for identifying the fungal elements in tissues. It demonstrates the polysaccharide content of fungal cell wall. Liberated aldehyde group from carbohydrate present in fungal cell wall, reduces methenamine silver nitrate complex and appears as brown-black stain over fungal cell wall. Bacteria are also stained black with this stain. A light green counter stain produces a pale, transparent background against which brown-black hyphae are easily recognized. It stains both live and dead fungi. It also stains higher filamentous bacteria like *Actinomycetes, Nocardia, Streptomyces* and *Actinomadura*. It can also be used to re-stain the corneal scrapes which have been stained previously with other stains. Sensitivity of this varies from 56-86% in culture proven cases; being higher to other stains like 10% KOH wet mount, Gram's stain and Giemsa stain.

Periodic Acid Schiff (PAS) stain: It is a selective stain, used for fungal identification. It is based upon Feulgen reaction in which Periodic acid oxidizes the polysaccharide of fungal and bacterial cell wall and in turn produces aldehyde. This re-colour the Schiff reagent and produces magenta coloured compound with blue stained nuclei. Proteins are unstained with this dye. This stain is useful in demonstrating fungi in clinical samples. Fungal elements can be readily recognized even in the thick clump of epithelial cells. It can also be used in a KOH wet mount which has been confirmed as negative for fungal hyphae. This offers maximum utilization of limited corneal material available for immediate analysis. Sensitivity of PAS staining in culture proven cases is as high as 91% (Xie *et al*., 2001). Gridley's stain is a modification of PAS technique where chromic acid is used as an oxidizing agent. It stains mycelia, yeast and elastic tissues as purple colour with yellow background. It also stains connective tissue and mucin that interfere in fungal identification. (J Chander, 3rd ed)

Calcofluor white stain: Calcofluor white is a nonspecific flourochrome stains which is being widely used for detection of fungal elements (Sutphin *et al*., 1986). It is a water soluble, colourless textile dye and fluorescent whitener. It selectively binds to β1-3 β1-4 polysaccharide such as chitin and cellulose of the fungal cell wall and when exposed to long wavelength ultraviolet rays or short wavelength visible light it fluoresces as apple green or bluish white depending on the filter which has been used (Fig:C). The fluorescence is easily detected by fluorescent microscope. It is a rapid two step process in which fungal hyphae and yeast cells are clearly delineated against a pale green background, even in small number or in thick smear. Appropriate filter is necessary to examine the corneal scrape stained with this stain. Sensitivity of Calcofluor white stain is 80-100% in culture proven mycotic keratitis. It can detect fungi in 50% of the smears previously considered negative by Gram's and Giemsa staining method. It is more sensitive than KOH wet mount in detecting the common ocular fungi. Parthasarathi *et al*., 2012 reported that sensitivity of CFW stain in detecting fungal corneal ulcer is 100% in culture proven mycotic keratitis (Parthasarathi *et al*., 2012).

Acridine orange: It is a simple fluorescent stain used to detect fungi in histological section of suspected mycotic lesion. It can be used as a rapid stain for smear and scraping as well as with KOH in wet mount preparation with fluorescent microscope. The fungal wall appears as a brilliant yellow-orange hue against a dark background. Another advantage of this stain is that it avoids misinterpretation of the artefacts such as vegetable fibers. Also certain bacteria, which has similar filaments like fungi e.g. *Nocardia* do not accept the strain at all and hence such filamentous bacteria can be easily differentiated from fungi by this stain (Kanungo *et al*., 1991).

Culture: It is considered as gold standard for diagnosis of fungal infection. Culture should be done on freshly prepared Sabouraud's Dextrose Agar (SDA) and Blood Agar. After

collection of appropriate sample, it should be inoculated on SDA in "C" shape pattern. Growth occurring within the streak lines should be considered as significant as fungi are the commonest laboratory contaminants. Liquid media (BHI) should also be inoculated to enrich the fungal element.

Sabouraud's Dextrose Agar: It is the most commonly used media for fungal cultivation. Media is made selective by addition of antibacterial antibiotics such as chloramphenicol (40µm/ml) or gentamicin (50µm/ml) as these substances inhibit the concomitant bacterial pathogens. Cyclohexamide should not be incorporated in media as it inhibits most of the fungi implicated in ocular fungi like *Aspergillus, Penicillium marneffei* and *Scytalidium* spp. Plates are incubated at 25^0C in BOD incubator.

Liquid brain heart infusion broth: It is used as add-on to solid media as it results in enrichment of fungal element, but if there is contamination, it is difficult to differentiate. Using these differential media, growth of fungi could be identified within 2 days in 54%, within 3 days in 83%, and within 1 week in 97% of patients with fungal corneal ulcer (Rosa *et al*., 1994). Culture growth is examined daily in the 1st week of incubation and then weekly till 4th week. If there is no growth even after 4 weeks, then it is presumed to be negative for fungal pathogens. Fungal isolates are identified by their colony characteristics and microscopic morphology in LCB wet mount and finally by fungal slide culture. Yeast isolates are identified by Gram's stain, germ tube production, chlamydospore formation, sugar fermentation and sugar assimilation.

Corneal biopsy: Even if the staining and culture is negative and still there is high clinical suspicion of fungi corneal biopsies is taken. Care is taken that the cornea do not perforate and therefore the biopsy is not taken where the cornea is very thin. It can be also taken during keratectomy or from corneal button. Histo-pathological examination reveal fungal elements which are parallel to collagen fibers. Loss of corneal epithelial cells with presence of nonspecific inflammatory cells with granuloma formation with stromal abscesses is also observed during histo-pathological examination.

Newer diagnostic tools in the diagnosis of Keratomycosis: Recently, newer techniques have been developed for prompt and reliable diagnosis having high sensitivity and specificity. Few of them are:

Polymerase Chain Reaction: It is the most sensitive and specific test to detect a specific DNA sequence. It can also be used for early diagnosis in impending perforation in suspected fungal keratitis. Even minute quantity of fungal DNA can be amplified by this technique. It is a useful technique especially in culture negative keratomycosis patients. Alexandrkis *et al*., used PCR for diagnosis of *Fusarium* keratitis in animal model, with a primer directly against the *Fusarium cutinase* gene. In his study, he had a sensitivity of 89% compared to 21% for culture (Alexandrkis *et al*., 1998). Usually PCR is used to detect segment of fungal specific DNA coding for *18sRNA gene, chitin synthetase gene* and cytochrome P-450L_1A_1 in clinical specimen (Embong *et al*., 2008; Dhib *et al*., 2012; Lai and Kirsch, 1989; Burgener-Kairuz *et al*., 1994). Consuelo Ferrer *et al*., reported that PCR has a sensitivity of 92.6% in detecting in fungal corneal ulcer compared to 66.7% and 59.3% sensitivity for stains and culture respectively. The time taken for PCR assay was 4-8 hr whereas culture took 1-35 days (Consuelo Ferrer, Jorge 2011). Sujit Vengayil *et al*; reported a 50%positive rate for identification of fungal filaments in PCR, 25% in culture, 40% in KOH wet mount and 35% in Gram's staining, in 40 presumed cases (30 untreated) of keratomycosis. In his study, sensitivity of PCR, KOH and Gram's stain in respect to culture was 70%, 60% and 40% respectively while the specificity was 56.7%, 66.7% and 66.7% respectively. In 10 of 40 eyes pre-treated with antifungal agents, PCR was positive in 50% but culture only in 30%. Time taken in this study for PCR was 4-8 hrs, whereas for culture was more than 5-7 days (Sujit Vengayil *et al*., 2009). It is however almost impossible to differentiate between pathogenic and contaminant fungi in PCR testing. PCR also fails to differentiate between live and dead fungi.

Confocal microscopy: It is a new non-invasive in-vivo method of imaging human cornea (Cavanagh *et al*., 1993). It is a rapid and sensitive diagnostic tool that can be used for early diagnosis and follow up of fungal keratitis (Chew *et al*., 1992; Avunduk *et al*., 2003). In-vivo imaging is important in culture negative cases of mycotic keratitis, as in about one-third patients with keratomycosis, culture was negative for fungi (Liesegang and Forster, 1980; McDonnell *et al*., 1992). Confocal microscopy can detect fungal hyphae even on 2nd day of fungal corneal ulcer infection. This is a great advantage as the delay in diagnosis and treatment is avoided which prevents loss of vision. Since this is an in-vivo technique corneal scraping is not required. The use of confocal microscopy as a diagnostic tool has conflicting viewpoints. Using a positive tissue diagnosis as the gold standard, Hau *et al*; did not recommend single use of confocal microscopy for the diagnosis of microbial keratitis (Hau *et al*., 2010). Conversely, Vaddavalli *et al*; reported that confocal microscopy provides accurate and reliable diagnosis in microbial keratitis, particularly when the corneal infiltrate is deep seated or patients are on treatment or when microbial keratitis develops after intra-corneal implants (Vaddavalli *et al*., 2011).

Anterior segment Optical Coherence Tomography: It provides detailed cross-sectional images of cornea with an axial resolution of upto 5 microns and a transverse resolution of 15 microns. Spectral domain optical coherence tomography is better for detection of stromal infiltration and corneal thickness in microbial keratitis (Wojtkowski *et al*., 2002). Localized small or full thickness large stromal cystic spaces (necrotic stroma) are unique patterns for fungal keratitis. Other features like epithelial defect, stromal edema, loss of all corneal layers except Descemet membrane and hyper-reflective stromal lesion are associated with both bacterial and fungal keratitis (Soliman *et al*., 2013).

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