ISOLATION AND IDENTIFICATION OF BACTERIAL AND FUNGAL AGENTS IN PATIENTS WITH CORNEAL ULCER IN A TERTIARY CARE OPHTHALMIC HOSPITAL

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BRANCH – IV

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CERTIFICATE

This is to certify that this dissertation titled **"ISOLATION AND IDENTIFICATION OF BACTERIAL AND FUNGAL AGENTS IN PATIENTS WITH CORNEAL ULCER IN A TERTIARY CARE OPHTHALMIC HOSPITAL"** is a bonafide record of work done by **Dr. E. KAYALVIZHI,** during the period of her Post graduate study from June 2006 to March 2009 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai-600003, in partial fulfillment of the requirement for **M.D. Microbiology** degree Examination of The Tamilnadu Dr. M.G.R. Medical University to be held in March 2009.

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DECLARATION

I declare that the dissertation entitled ***"ISOLATION AND IDENTIFICATION OF BACTERIAL AND FUNGAL AGENTS IN PATIENTS WITH CORNEAL ULCER IN A TERTIARY CARE OPHTHALMIC HOSPITAL"*** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***May 2007 to May 2008*** *under the guidance of Professor* ***Dr.S. GEETHALAKSHMI M.D.,Ph.D.,*** *Vice Principal, Professor of Microbiology, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to the Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Microbiology (Branch IV) examination to be held in March 2009.*

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Date :

Signature of the Candidate
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CONTENTS

INTRODUCTION

Eyes are our main contact with the world. Eyesight is the sense that everyone values more than all the rest. Light waves from an object enter the eye first through the cornea, the transparent dome which serves as the outer window of the eye[34].

Corneal ulceration is defined as a loss of corneal epithelium with underlying stromal infiltration and suppuration associated with signs of inflammation[6]. Corneal blindness is a major public health problem worldwide and infectious keratitis is one of the predominant preventable cause[4].

In south East Asia according to an estimate, 6.5million people are affected with, and 1.3 million eyes become blind due to corneal ulcer every year[72]. Corneal ulceration is common in South India, and most often occurs after a superficial corneal injury with organic material.

Any known organism can cause microbial keratitis and corneal ulceration, given the appropriate conditions and predisposing risk factors. A wide spectrum of microbial organisms like bacteria, fungi, viruses and parasites can produce infectious corneal ulcer. The most commonly encountered aetiological agents involved show tremendous geographical variance[58].

Corneal ulcer is an ocular emergency that requires prompt management to ensure the best visual outcome for the patient. A clinical diagnosis does not give an unequivocal indication of the causative organism because a wide range of organisms can produce a similar clinical picture. Therefore microbiological evaluation plays a significant role in the diagnosis and treatment of corneal ulcer.

Direct microscopic evaluation of smears provide immediate information about the causative organism and is helpful in starting antimicrobial therapy, but culture of microbial pathogens is considered to be the gold standard[5].

Given the rapid progression and virulent nature of many infectious agents, any corneal inflammation should be considered as a threat to vision, requiring prompt treatment and the empirical therapy is based on the prevailing antibiotic susceptibility profiles, which can be subsequently modified based on the culture and sensitivity results.

The incidence of fungal keratitis has increased in the last few years due to the improvement in microbiologic diagnostic techniques and introduction of new therapeutic measures such as widespread use of broad spectrum antibiotics, and immunosuppressive drugs[4].

Antimicrobial susceptibility tests are mandatory to monitor the efficiency

of available antimicrobial agents and the emergence of drug resistance among bacterial and fungal isolates.

Considering the importance of corneal ulceration and its impact on vision, the present study was conducted to identify the predisposing factors of corneal ulcers, the aetiological agents and their susceptibility profiles in patients attending a tertiary care Ophthalmic hospital in Chennai.

AIMS OF THE STUDY

- To isolate and identify the fungal and bacterial aetiological agents of Infectious corneal ulcer.
- To find a correlation between aetiological agents of corneal ulcer and age, sex and occupation of the patients.
- To identify the predisposing factors for fungal and bacterial corneal ulcer.
- To evaluate the sensitivity pattern of bacterial isolates from all cases to the commonly used antibiotics.
- To study the sensitivity pattern of fungal isolates to the commonly used antifungal drugs.

REVIEW OF LITERATURE

'Early detection means vision protection'

Corneal ulceration in the developing world is a silent epidemic. Corneal infection is a leading cause of ocular morbidity and blindness worldwide[21].

CORNEA:

The cornea is the transparent, anterior portion of the outer shell of the eye, corresponding to a watch crystal. The cornea consists of five layers[34]

1. Nonkeratinised stratified squamous epithelium
2. Bowman's layer
3. Stroma
4. Descemet's membrane
5. Endothelium

Cornea has four major functions

1. Transmission of light by its transparency
2. Refraction of light
3. Maintenance of structural integrity of the globe
4. Protection of the eye from infective organisms

To accomplish these functions cornea must maintain its transparency.

The transparency of cornea is due to

1. Avascularity and absence of lymphatics.
2. Uniform refractive index of all the layers.
3. Uniform spacing of the collagen fibrils in the stroma.
4. Relatively dehydrated state of the corneal stroma.

Nutrition of the cornea is dependant on glucose diffusing from aqueous humor and oxygen diffusion through tear film. Cornea is an extremely sensitive structure owing to its dense nerve supply[74].

The tear film containing lysozyme, lactoferrin, beta-lysin, secretary IgA, combining with the mechanical action of the blinking eyelids, serve as a defense against microbial attachment[58].

HISTORY:

It is important to take note of the first description of important pathogens. In 1879, Leber was credited with documenting the first case of fungal keratitis in a farmer, which was due to Aspergillus glaucus[107]. Neisser was credited with discovering the first ocular bacterial pathogen. In 1881 the first Pseudomonas Corneal Ulcer was described.

Aspergillus species were the predominant fungi most commonly isolated from mycotic corneal ulcers up to 1960. By 1998, some 105 species in 56 genera of fungi had been reported as causes of mycotic corneal ulcer[107].

In 1974, Naginton et al first described Acanthomoeba Keratitis. Since then, this organism has become a well recognized case of corneal

infection[21].

AETIOLOGICAL AGENTS

The aetiological agents involved in corneal ulceration can be classified as[20]

1. Bacterial
2. Fungal
3. Viral and
4. Protozoal

1. Bacterial

a. Gram positive cocci
    i. Staphylococcus aureus
    ii. Staphylococcus epidermidis
    iii. Streptococcus pneumoniae, Streptococcus pyogenes,
    iv. Streptococcus viridans
    v. Enterococcus faecalis
    vi. Peptostreptococcus spp

Gram positive Bacilli
    vii. Bacillus species
    viii. Corynebacterium species

b. Gram negative Bacilli
    i. Pseudomonas species
    ii. Hemophilus influenzae
    iii. Proteus mirabilis
    iv. Serratia marcescens

v. Escherichia coli
vi. Klebsiella pneumoniae
vii. Aeromonas hydrophila
viii. Morganella morganii

c. Gram negative cocco bacilli
    i. Neisseria gonorrhoeae
    ii. Moraxella lacunata
    iii. Acinetobacter species

d. Mycobacteria

e. Actinomycetes

f. Nocardia sp.

g. Treponema pallidum

h. Chlamydia trachomatis

i. Those invading intact eye
    i. Neisseria gonorrhoeae
    ii. Corynebacterium diphtheriae
    iii. Haemophilus species
    iv. Listeria monocytogenes

Any bacteria can potentially cause keratitis. The causative organism cannot be identified from the clinical features. The relative frequency of different bacteria as causative agents in keratitis may vary geographically[59].

There has also been a change in the spectrum of bacteria causing keratitis with time[87]. Staphylococcus species, Pseudomonas species and Streptococcus species appear to be the predominant causes of bacterial

corneal ulcer in United States[39].

Similarly common bacterial pathogens in most of the studies in India are Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas species[57].

In older reported series, Streptococcus pneumoniae was the most common cause of bacterial keratitis. Its prevalence as an aetiological agent has been superseded both by Staphylococcus species and by Pseudomonas species[50].

Although relatively uncommon, keratitis caused by E.coli, Klebsiella pneumoniae, Acinetobacter species and Serratia marcescens have been documented[50].

Non ulcerative keratitis such as interstitial keratitis is caused by Treponema pallidum (congenital and acquired syphilis). Other causes are Mycobacterium tuberculosis, Mycobacterium leprae, Borrelia burgdorferi infections and relapsing fever[82].

2. Fungal

Fungi causing keratitis belong to at least 56 genera[107]. Most common fungi causing keratitis belong to members of the genus

a. Aspergillus

b. Fusarium

c. Candida

Common fungal agents implicated in corneal ulcers[40]

Phaeoid Hyphomycetes

a. Aureobasidium pullulans
b. Alternaria species
c. Bipolaris species
d. Curvularia species
e. Exserohilum species
f. Cladosporium species

Hyaline Hyphomycetes

a. Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger
b. Acremonium species
c. Penicillium species
d. Fusarium species
e. Paecilomyces species
f. Pseudallescheria boydii

Yeast like Fungi

a. Candida albicans
b. Candida tropicalis
c. Candida krusei

Mycotic corneal ulceration although not reported as a major cause of keratitis in developed countries is an important cause of ulcerative keratitis in tropical regions including India[86].

Mycotic ulcers of the eye usually occur subsequent to trauma to the cornea by vegetative matter, soil or surgery. The majority of the fungal organisms involved are soil saprophyte whose airborne conidia find the

injured tissue a suitable environment for growth.

The list of fungal species associated with such infections is very long, however a few species seem to be more aggressive opportunists and account for the majority of infections recorded[83].

Mycotic corneal ulcer is characterized by a raised epithelium with a white shaggy border, the fuzzy hyphate border extends beyond the ulcer edge and may form satellite lesions[44]. Unless eradicated rapidly, the lesions of mycotic keratitis advance into the deep stroma of the cornea, penetrate through the descemet's membrane leading to perforation of the cornea and invasion of the internal orbit[65]. Hence fungal keratitis carries a bad prognosis if not treated promptly and accurately.

Various studies in developing countries have revealed that this important ophthalmologic problem is responsible for 6 to 55 percent of all ulcerative cases investigated[103].

Species of Aspergillus genus especially Asp.fumigatus, Asp. flavus and Asp. niger appear to predominate in studies conducted in most parts of India[107]. All the Aspergillus species are common saprobes in the soil and on decaying plant material and their spores are ubiquitous in the environment. The human beings are regularly exposed to these spores, particularly people working with the decaying vegetations like moldy hay in agriculture. Minor trauma to corneal epithelium leads to direct implantation of the fungal spores leading to fungal ulcer.

Aspergillus species are hyaline saprophytic filamentous fungi which grow readily on Sabouraud's dextrose agar. The colonies of Asp.fumigatus are velvety or powdery, smoky green with white to tan reverse. The conidiophore is smooth with uniseriate phialides covering upper half of the vesicle. The colonies of Asp. flavus are velvety, yellow to green in colour. The phialides are uniseriate or biseriate but cover the entire vesicle. The colonies of Asp.niger are woolly at first, white to yellow, then turning dark brown to black later. Phialides are biseriate, covering the entire vesicle[40].

Some studies in South India have reported Fusarium species to be more common than Aspergillus species. Fusarium species have also been found to be the principal fungal pathogen in Florida, Paraguay, Singapore, Nigeria, Tanzania and Hong Kong. This phenomenon may be explained by differences in climate and the natural environment[26].

Species of Penicillium, Alternaria, Curvularia, Bipolaris, Acremonium, Aureobasidium were isolated frequently in studies conducted in most parts of India and Nepal[11,45].

Candida albicans was the most common agent in a study conducted at Will's eye hospital, Philadelphia in May 2000[101].

Prevalence of bacterial coinfection in mycotic corneal ulcer ranged from 5 to 30 percent in various studies conducted in India and South America[75]. The risk of bacterial co-infection was 3.2 times greater with

yeast keratitis than with filamentous fungal keratitis in a study conducted in United States[75].

3. Viral

a. Herpes simplex virus
b. Varicella zoster virus
c. Adeno virus
d. Epstein Barr virus
e. Rubella virus
f. Entero virus
g. Cox sackie virus

Viral keratitis usually presents as a non suppurative superficial keratitis of the viral agents. Herpes simplex virus and Adeno virus are most commonly involved, producing punctate epithelial erosions. More rarely viruses associated with vaccinia, measles, mumps, herpes zoster and infectious mononucleosis may affect the cornea.

In industrialized countries, Herpes simplex virus keratitis is said to be the most frequent single cause of corneal opacities and subsequent visual disability and blindness, primarily because of its recurrent nature[53]. By contrast in developing countries where prevalence of bacterial and fungal corneal ulcers and associated ocular diseases are high, herpetic keratitis, although known to be present is generally regarded as a less significant ophthalmic problem[2].

4. Parasites

a. Acanthamoeba polyphaga, Acanthamoeba castellanii
b. Onchocerca volvulus
c. Leishmania brasiliensis
d. Trypanosoma species
e. Encephalitozoon species
f. Nosema species
g. Vittaforma corneae

Acanthamoeba species are ubiquitous free living protozoan parasites that can be isolated from diverse habitats like soil, stagnant water and fresh water ponds[76].

Acanthamoeba species can infect immunocompromised as well as healthy persons. Keratitis is the most common human infection caused by Acanthamoeba species. Acanthamoeba keratitis is a severe progressive sight threatening infection of cornea [21]. Since the first Acanthamoeba keratitis was described in 1974, more than 200 cases have been reported world wide. The species commonly implicated are Acanthamoeba polyphaga, Acanthamoeba castellanii and Acanthamoeba culbertsonii[63].

Acanthamoeba keratitis is frequently associated with contact lens wear. In developed world, up to 93 percent are related to contact lens use[79].

It has been identified that even non contact lens wearers are prone to this infection[91].

The fall of dust particles, trauma due to vegetable matter, contact with contaminated water etc have been found to be the predominant risk factor of Acanthamoeba keratitis. The isolation rate is 1.1 percent among clinically suspected cases[59].

PREDISPOSING FACTORS:

There are multiple predisposing factors of which ocular trauma is the most common.

1. Ocular trauma:

Ocular trauma particularly with vegetable matter is a well known predisposing factor for fungal, bacterial and protozoal corneal ulceration[54,78].

Often the traumatic episode that causes a break in the epithelium is minor and goes unnoticed, it may be nothing more than a minute abrasion caused by air borne debris or a small foreign body[51].

2. Contact lens wear:

Contact lens wear appears to be the most important risk factor for the development of ulcerative keratitis in developed countries[19].

In several studies from United States, Pseudomonas species was reported to be the most commonly isolated organism in corneal ulcers associated with contact lens wear[35].

Many studies have reported well recognized association of contact

lens wear with Acanthamoeba keratitis and fungal corneal ulceration[99,102].

3. Age and Occupation:

A high prevalence of microbial keratitis is observed in the actively working age group (20- 40yrs), particularly among agricultural workers from rural background[97].

Other risk factors:

4. Use of topical corticosteroids[4]
5. Previous ocular surgery like keratoplasty[81]
6. Ocular surface disease[54]
7. Absence of corneal sensation from herpetic keratitis[80]
8. Systemic diseases such as diabetes mellitus[54]

PATHOGENESIS

The adherence of bacteria to the disrupted or normal corneal epithelium is the main event in the pathogenesis of corneal ulcer. Many bacteria display several adhesins on fimbriae and nonfimbriated structures. Such adhesive proteins recognize receptors on host cells and promote bacterial entry. They also activate leukocyte migration and induce cytokine production[69]. Once the bacteria has adhered to the corneal epithelial surface, stromal invasion is facilitated by proteinases that degrade basement membrane and extracellular matrix. Number of exotoxins, elastases, proteases, and heat stable hemolysins contribute to the pathogenesis[106].

Fungi gain access into the corneal stroma through a defect in the epithelium. They multiply and cause tissue necrosis and elicit inflammatory reaction. They can penetrate the intact descemet membrane and gain access into the anterior chamber or the posterior segment resulting in true exogenous endophthalmitis. Mycotoxins and proteolytic enzymes of fungi augment the tissue damage[25].

Acanthamoeba species enter through minor abrasions in the cornea produced by contact lens or external injury. In the cornea it elicits inflammation with hypopyon formation. Further progression of infection leads to perforation[70].

Herpes simplex viral keratitis appears as a corneal ulcer or dendritic keratitis or as vesicles of the eye lids. Recurrent lesions of the eye are common with Herpes simplex keratitis. There may be progressive involvement of the corneal stroma with permanent opacification and blindess[41].

CLINICAL MANIFESTATION

Clinical presentation and examination findings of corneal ulcers differ according to aetiological agents involved. All ulcers generally present with complaints of

- Pain
- Watering
- Redness
- Photophobia and

- Diminished Vision

On examination there may be conjunctival chemosis, congestion, purulent discharge, hypopyon and stromal infiltration[34,80].

In addition, the presenting clinical features that are specific to fungal ulcers include a greyish white infiltrate with feathery margins, rough texture and raised borders with endothelial plaques and satellite lesions. A deep stromal infiltrate with an intact epithelium may also be present[25].

In parasitic ulcers, especially caused by Acanthamoeba, there may be photophobia and severe pain, often out of proportion to the clinical appearance. Early infection manifests as diffuse epithelial keratitis, dendritic lesion or radial keratoneuritis. The classic ring infiltrate is a late finding in parasitic ulcers[58].

The different types of clinical presentation in Herpes simplex viral keratitis include dendritic ulcers, geographic ulcers, with stromal keratitis, endothelitis and kerato uveitis[2].

DIAGNOSTIC TECHNIQUES

Many fungal ulcers demonstrate no striking morphologic pattern, and often it is not possible to differentiate clinically between fungal keratitis and bacterial keratitis[25].

To determine the causative organism meticulous collection of microbiological specimens is of critical importance. The corneal ulcer is scraped for microscopy, culture and for further investigations if indicated[67].

MICROSCOPIC EVALUATION OF SMEARS

A. Potassium Hydroxide(KOH) / Wet mounts

a) Wet Mount

Samples of corneal scraping should be touched, not smeared, on the slide and examined under light microscope under reduced light for the presence of motile Acanthamoeba trophozoites. Accurate diagnosis of Acanthamoeba can be established with the aid of direct wet mount examination of corneal scraping. Wet mount is effective for observing the motile trophozoites of Acanthamoeba[46].

b) 10% KOH Mount

Corneal scraping are placed over a clean glass slide, a drop of 10% potassium hydroxide reagent is added over the specimen and a coverslip is placed over it taking care to avoid trapping of air bubbles. Proteinaceous components, such as host cells are partially digested by the alkali, leaving the intact polysaccharide containing fungal cell walls[93].

In 1985 Araffa et al reported that ink KOH staining was as effective, much easier and less expensive than calcofluor white staining for detection of fungi in corneal tissue[3].

Sharma et al in 1990 reported that 10% KOH mount could be used to demonstrate Acanthamoeba cysts in corneal scrapings there by permitting rapid presumptive diagnosis of Acanthamoeba keratitis[94].

In 1993 Vajpayee et al reported that 10% KOH Wetmounts

demonstrated fungus in 94.3% of total culture proven cases of keratomycosis[108].

In 1998, Sharma et al have reported that KOH preparations demonstrated fungus in 100 percent of total culture proven cases[93].

Chowdhary et al in 2005 have concluded that the direct microscopic examination of KOH mount is a rapid, reliable and inexpensive diagnostic modality, which would facilitate the institution of early antifungal therapy before culture results become available, thus proving to be sight saving[13].

In 2007, Bharathi et al concluded that a potassium hydroxide smear is of greater diagnostic value in the diagnosis of fungal keratitis, Nocardia keratitis and Acanthamoeba keratitis[5].

STAINS

a.Gram's and Giemsa Stains:

Gram's stain and Giemsa stain preparations are widely used for the rapid detection of microbes. Gram's stain study enables the clinician to start empirical treatment[67].

For Gram's stain preparation, the specimen is smeared evenly over a clean slide, fixed with 95% methyl alcohol for 5 minutes and stained by the standard gram's staining method. It can confirm the presence of microorganisms with a sensitivity of 55-79%. It can also distinguish gram positive and gram negative bacteria from fungi[80].

In 2006, Bharathi et al reported 100% sensitivity of Gram's stain procedure in the diagnosis of Bacterial Keratitis[5].

Fungus was identified in 86.4% percent of cases with Gram's stain preparation in the study by Sharma et al in 1998[93].

Giemsa stain also is useful to distinguish bacteria, fungi and Acanthamoeba. Chlamydia inclusion bodies can also be identified with Giemsa stain[80].

b.Lactophenol Cotton Blue stain:

Corneal scrapings are placed over a clean glass slide and drop of Lactophenol cotton blue reagent is added over the specimen and a coverslip is placed taking care to avoid trapping of air bubbles. It was found to be effective for demonstration of fungal structures and Acanthamoeba cysts in corneal scrapings.

Lactophenol Cotton Blue mount was positive in more than 80% of culture positive cases of keratitis due to Fusarium Sp and Aspergillus species and in more than 75% of culture proven cases of Acanthamoeba keratitis[104].

c.Calcofluor white stain:

This is a water soluble colourless textile dye and fluorescent whitener. It selectively binds to chitin and cellulose of the fungal cell wall. It fluoresces light blue when exposed to UV light (346-365nm)[40].

To the corneal scrapings in a slide, 1 drop of 0.1% Calcofluor white with 0.1% Evan's blue and 1 drop of 10% KOH are added. A coverslip is placed over the specimen and examined under fluorescent microscope.

The morphology of smaller fungal filaments was better appreciated in Calcofluor white mount than KOH mount[93].

Chander et al in 1993 reported that fungi could be detected in corneal tissue by Calcofluor white staining in 95.2% of patients, where as KOH mounts and culture were positive in only 71.4% of patients[11]. Calcofluor white stain is also useful in detecting Acanthamoeba cysts in specimens under a fluorescent microscope[109].

d.Acridine orange stain :

Acridine orange dye has an affinity for nucleic acid. When fungi are stained with this dye, RNA component of the cell fluoresces with shades of orange red and DNA component fluoresces green under fluorescent microscope.

Acanthamoeba cysts fluoresces bright yellow to orange. This stain has been used for direct examination of corneal scrapings in cases of Acanthamoeba keratitis[40].

Other stains:

Special stains include Ziehl-Neelsen acid fast stain for the identification of Mycobacterium species and Kinyoun stain for Nocardia species[87].

A fluorescent Gram's stain was reported to be useful in screening corneal scrapings for microbes[37].

Special techniques:

Liesegang et al in 1980 reported that the limulus lysate assay was an effective means in establishing the presence of Gram negative bacilli in corneal tissues. However the cost of the assay is prohibitively expensive for routine use[52].

The use of fluorescein conjugated lectins provides rapid and species specific detection of fungi in corneal tissue[104].

In 1989, Robin et al have reported the use of fluorescein conjugated lectins, which bind to both the trophozoites and cysts forms of Acanthamoeba[84].

CULTURE:

Microbial culture is considered to be the gold standard in the detection of causative organisms of corneal ulcer[61].

Collection of sample:

Corneal scrapings are collected under strict aseptic precautions by an ophthalmologist using a sterile number 15 Bard Parker blade after instillation of a local anaesthetic like 2% lignocaine hydrochloride.

Materials obtained from scraping the leading edge and base of each ulcer was smeared onto two slides for direct microscopic examination and inoculated onto culture medias[50].

Processing: (inoculation into medias):

Each scraping was used to inoculate one medium at a time, in Mac conkey agar plate and Blood agar plate, in rows of ‘C’ shaped streaks.

Scrapings were also inoculated into Sabourauds dextrose agar slants and Brain heart infusion broth[67].

For isolation of Acanthamoeba, Non nutrient agar with E.coli overlay was used. This medium contained 20-25 ml of 1.5% agar in distilled water (without nutrients). A loopfull of heavy suspension of E.coli from 24-48 hour culture in blood agar plate was spread in an area of 1 cm diameter in the centre of the plate. The bacteria act as the food source for trophozoites and the non nutrient agar prevents over growth of the bacteria.

Inoculum was placed on the top of the bacterial spot. The plate was then sealed with tape to prevent drying, incubated at 37°C and examined after18-24 hours with agar side up under 10 x objective. If no amoeba were seen, plates were incubated further for 10 days and examined daily[23].

Interpretation of growth:

Microbial cultures were considered significant

- If growth of same organism was observed on more than one solid media.
- If there was confluent growth at the site of inoculation on one solid medium.
- If growth in one medium was consistent with direct microscopic findings.
- If the same organism was grown from repeated scrapings[67].

Bacterial:

The specific identification of bacterial pathogens was based on the microscopic morphology, staining characteristics and biochemical properties using standard laboratory criteria[1]. Any organism out side the 'C' streak was considered a contaminant and not further processed[34].

Fungal:

Fungal isolates were identified by studying the colony morphology, colony colour, production and arrangement of conidia in preparations stained by Lactophenol cotton blue stain[24].

When identification was difficult due to inadequate sporulation, Riddle's slide culture technique was employed[23].

Protozoal:

Acanthamoeba would appear as small refractile bodies that migrate from inoculum leaving irregular trails or tortuous tracks through the bacteria.

Trophozoites of Acanthamoeba are 14-15 micrometer, actively motile at 37 degree centigrade. They have numerous spiny acanthopodia. Cysts are smaller 10-25 micrometers, double walled, with wrinkled outer wall (ecto cyst) and a stellate polygonal inner wall (endo cyst).

Identification beyond genus level requires species specific immunological reagents which should be confirmed by isoenzyme profiles and restriction fragment length polymorphism using mitochondrial DNA[43].

ANTIMICROBIAL SUSCEPTIBILITY TESTING:

As resistance patterns to commonly used ophthalmic antibiotics and antifungals continue to shift, sensitivity testing play an important role both in, appropriate management of individual cases based on susceptibility characteristics and for community surveillance[67].

ANTIBACTERIAL SUSCEPTIBILITY TESTING:

Antibiotic sensitivity testing was performed by the Kirby-Bauer disc diffusion technique, using 0.5 McFarland's turbidity as the standard inoculum's density on Mueller Hinton agar plates. Commercial Hi-Media antibiotic discs were used[42].

ANTIFUNGAL SUSCEPTIBILITY TESTING:

The recent increased incidence of fungal infections and the growing number of new antifungal agents have multiplied the demand and interest for invitro antifungal susceptibility testing[1].

Antifungal susceptibility testing was performed by[64]

Agar based methods:

1. Agar dilution method
2. Disc diffusion method
3. E-test method

Broth based methods:

1. Broth macrodilution method
2. Broth microdilution method
3. Colorimetric methods.

Broth micro dilution method:

The clinical and laboratory standards Institute (CLSI) subcommittee on Antifungal Susceptibility tests has developed a reproducible procedure for antifungal susceptibility testing of filamentous fungi by a broth microdilution format; the M38-A document for Filamentous fungi. It recommends the use of RPMI-1640 medium with glutamine, without bicarbonate and with phenol red as a pH indicator supplemented with 0.2% glucose and buffered to a pH of 7.0 with 0.165 mol/L MOPS (3-N-morpholino propane sulfonic acid) as used in M27– A_2 standard for yeasts.

Inoculum preparation of conidial or sporangiospore suspensions must be adjusted using a spectrophotometer in the range of 0.4 x 10^4 to 5 x 10^4 CFU/ml to get the most reproducible MIC data. The optical density (OD) at 530 nm required for Aspergillus species is 0.09 – 0.11; for Fusaruim species, 0.15 – 0.17. A small drop of Tween 20 as wetting agent is added to facilitate the preparation of Aspergillus inocula[15].

Standard two fold serial dilutions across the concentration range to be tested are made. In broth dilution method, a total of 100 microlitres of broth containing antifungal agents in two fold dilutions is placed in duplicate wells of sterile 96 well microtitre plates.

For MIC determination, the following range of drug concentrations are used

Amphotericin B = 0 – 16 µg/ml

Fluconazole = 0 – 512 µg/ml.

Broth macro dilution experiment was performed in sterile 6ml polystyrene tubes with a final volume of 1ml. Two times the required concentrations of the drug and the conidial suspensions are prepared by 2 fold serial dilutions[22].

Good agreement between results obtained by both Broth microdilution and macrodilution methods for moulds has been documented[30].

Limitations of Broth Dilution Method

The invitro susceptibility testing of fungi is till date a problem in laboratory due to difficulty in standardization.

- Long incubation period for obtaining inoculum(7 to 10 days)
- Problems of obtaining inoculum when moulds present germinate to hyphal forms and do not form conidia
- Poor growth of some species with the assay medium recommended for susceptibility testing
- Lack of correlation with clinical outcome[1]

Some studies have been conducted trying to overcome the limitations. Alternative assay media and modification of inoculum size, incubation time, reading procedure, and end point determination have been assessed[18].

Agar based methods are attractive because of their simplicity and low cost. More recently an agar diffusion method has been developed for

testing moulds by disc diffusion methodology with five antifungal agents – Amphotericin B, Itraconazole, Caspofungin, Voriconazole and Posaconazole.

A Neo-Sensitabs assay utilizing 9mm diameter tablets for the antifungal susceptibility testing of yeasts and other fungal pathogen with several antifungal agents was done by A Espinel–Ingroff and colleagues. He concluded that both tablet and disc assays were not always be able to differentiate intermediate from susceptible and resistant values for some species and antifungal agent combinations[28]

Agar based methods are not widely used for antifungal susceptibility testing since their clinical relevance have yet to be determined and breakpoints are not available for mould testing .

Other diagnostic methods

When corneal smears and culture are negative and the keratitis is not responding to empirical therapy, then a diagnostic keratectomy or a corneal biopsy is necessary to establish a diagnosis.

The corneal biopsy specimen should be submitted to the laboratory for smears and cultures. A substantial portion should be submitted for histopathological examination. Histopathological examination of corneal buttons can reveal the presence of fungal elements in 75% of patients[32,25].

RECENT METHODS

Impression cytology and confocal microscopy are other rare diagnostic tools. Confocal microscopy is a new and non invasive

procedure in which four dimensional view of internal structures are possible at cellular level[81].

MOLECULAR DIAGNOSIS:

PCR:

Polymerase chain reaction amplification can be used to detect the presence of as few as 10 organisms per 100 ml volume of clinical specimen.

In 2005, Manish Kumar et al., have reported, sensitive and rapid polymerase chain reaction based diagnosis of mycotic keratitis through single stranded conformation polymorphism in their study[60].

Corneal scrapings are processed for DNA extraction which is amplified by fungal specific primers of internal transcribed spacer region I (ITS1). The products are sequenced and analysed by single stranded conformation polymorphism (SSCP) for species identification[60].

Recently, Novel Real time PCR assays targeting the fungal ITS2 region (internal transcribed spacer region 2) are developed for the detection and differentiation of medically important Aspergillus species and Candida species using a light cycler instrument. This (test) assay might become an important tool in the early diagnosis of fungal infections in future[14].

PCR and sequence analysis of 16S rDNA has been used to detect bacterial pathogens in patients with keratitis with promising results[47].

Other methods of detections include:

- Detection of fungal antigen by ELISA and
- Detection of fungal metabolites (fungus specific metabolites) by gas liquid chromatography[40].

THERAPY

TREATMENT OF BACTERIAL CORNEAL ULCERS:

Topical antibiotic administration is the primary mode of therapy for bacterial keratitis. Sub conjunctival injection of antibiotics is considered only in severe cases with scleral extension[80].

Fortified preparations containing much higher concentration than commercial ones are recommended. Two fortified preparations administered concurrently provide a broad spectrum of activity[98].

Oral or parenteral antibiotics have been shown to be of no benefit and are indicated only in ulcers with perforation, scleral involvement or endophthalmitis. An exception is Gonococcal infections which require systemic ceftriaxone[66].

The topical ophthalmic agents available for bacterial keratitis include Fluoroquinolone, Erythromycin, Aminoglycosides like Tobramycin, Gentamycin, and Amikacin and Cefazolins. The most commonly used Cephalosporin in fortified drops is Cefazolin. The aminoglycoside antibiotics used in fortified drops are Gentamicin and Tobramycin.

Other antibiotics used in special occasions are Amikacin, Vancomycin, Methicillin, Cotrimoxazole, Clarithromycin and anti-Pseudomonas antibiotics such as Piperacillin, Ceftazidime, Cefoperazone and Imipenem[66].

Smitha et al, in 2005, reported high susceptibility of Pseudomonas sp to Fluoroquinolones and among Aminoglycosides, Gentamycin was found to be highly effective than Amikacin or Tobramycin[95].

The fourth generation Fluoroquinolne, Gatifloxacin has been shown to be superior to Ciprofloxacin for management of bacterial keratitis[73].

TREATMENT OF FUNGAL CORNEAL ULCERS:

Natamycin 5% suspension is the first choice for treatment of filamentous fungal corneal ulcers[69].

Amphotericin B is particularly effective against yeasts but less effective against filamentous fungi; therefore it is the first agent of choice against yeasts. The Azoles and Flucytosine are generally employed as alternative agents for advanced ulcers or for ulcers not responding to Polyenes[66].

Mohan et al in 1989, obtained success rate of 64.7%, when 1% Miconazole was used to treat smear positive keratitis[62].

Oral Fluconazole and Voriconazole have good intraocular penetration with few adverse effects compared to other azoles[25].

Newer agents such as triazoles (Posaconazole and Ravuconazole), Echinocandins, Sodarin derivatives and the Nikkomycins might improve the treatment of fungal keratitis in future[27].

TREATMENT OF ACANTHAMOEBA KERATITIS

It is difficult to treat Acanthamoeba keratitis. Chlorhexidine and Polyhexamethylene biguanide (PHMB) are most effective against trophozoites and cysts and are recommended as the first line therapy for Acanthamoeba keratitis. Medications have to be continued for 3 – 6 months after clinical resolution of infection to prevent relapses.

Other Compounds that have been useful include

Propamidene isethionate, . Pentamidine, Hexamidene disethionate, Dibromopropamidine. Aminoglycosides such as Neomycin also have been found to be useful.

Antifungal agents that have shown to be promising in vitro include oral ketaconazole, oral itraconazole, clotrimazole and miconazole[38].

SURGICAL TREATMENT OF CORNEAL ULCER

Frequent corneal debridement with a spatula is helpful which debulks fungal organisms and epithelium and enhances penetration of the topical antifungal agent[25].

Although the mainstay of initial management of severe infective keratitis remains aggressive antimicrobial therapy, the role of timely surgical intervention in the form of therapeutic keratoplasty should be

considered in patients with severe end stage diseases. The timing of surgery is critical. The surgery should be performed within 4 weeks of presentation. Therapeutic keratoplasty may effectively treat severe refractory infectious corneal ulcer[89].

MATERIALS AND METHODS

PERIOD OF STUDY

This is a cross sectional study undertaken over a period of one year from May 2007 to May 2008.

PLACE OF STUDY

This study was carried out at the Institute of Microbiology, Madras Medical College, Chennai and Regional Institute of Ophthalmology, Government Ophthalmic Hospital, Chennai.

STUDY GROUP

All patients presenting to the Outpatient Department of the Regional Institute of Ophthalmology Government Ophthalmic Hospital (RIOGOH), with signs and symptoms of infectious corneal ulcer such as pain, redness, watering of the eye, dimness of vision and photophobia were included in the study.

ETHICAL CONSIDERATIONS

Written consent to participate in the study was obtained from the patients or their guardians after providing full explanation of the study. This study was reviewed and approved by Institutional ethical committee, Madras Medical College & General Hospital, Chennai 3. All data were handled confidentially and anonymously.

STATISTICAL ANALYSIS

Statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) and Epi-Info softwares by a statistician. The proportional data of this cross sectional study were tested using Pearson's Chi Square analysis test and Binomial proportion test

SPECIMEN COLLECTION

Infectious corneal ulcer patients were sent to the Microbiology Department after detailed clinical examination by the Ophthalmologists for investigation. After getting informed consent, few drops of local anaesthetic like 4% xylocaine was instilled into the affected eye and scrapings were taken by means of a sterile number 15 Bard Parker surgical blade, from the leading edge and base of the ulcer.

Four sets of scrapings were taken and processed as follows[34]

1. First set of scraping was applied to 3 sterile microscopic slides for 10% Potassium hydroxide (KOH) mount preparation, Gram's stain procedure and Modified Ziehl-Neelsen staining.
2. Second set of scraping was inoculated onto solid media like blood agar and MacConkey agar by 'C' streak method.
3. Third set of scraping was inoculated onto 2 Sabouraud dextrose agar (SDA) slants devoid of antibiotics and cycloheximide.

4. Fourth set of scraping was inoculated into enrichment media - Brain heart infusion broth.

The Bard Parker blade was flame sterilized and cooled between each set of scrapings.

SPECIMEN PROCESSING

KOH mount preparation was examined for the presence of hyphal elements, conidial forms and for the presence of Acanthamoeba cysts[5].

Gram positive or Gram negative organisms or yeast cells were looked for in Gram's stain preparation.

Bacterial culture plates and the inoculated enrichment medium were incubated at 37°C for 48 hours. Subculture from the enrichment broth was made onto blood agar and MacConkey agar plates and incubated at 37°C for 48 hours. Inoculated SDA slants were incubated at 30°C for up to 4 weeks[83].

INTERPRETATION

Interpretation of Bacterial culture

Bacterial culture plates were observed for growth at 24 hours and 48 hours. According to Jones recommendation, if the number of colonies were 10 or more within the streak line on solid medium it was taken as significant bacterial growth. Any growth seen outside the 'C' streak was considered as contaminant[34].

Bacterial isolates were identified by means of Gram's staining, motility

and biochemical reactions by standard microbiological techniques as recommended by Clinical and Laboratory Standards Institute (CLSI).

All bacterial isolates were preserved in 0.2 – 0.5 % semisolid Nutrient agar slopes at 4 – 5° C in refrigerator with periodic subculture every 2 months[56].

Interpretation of Fungal culture

Inoculated SDA slants were incubated at 30°C for minimum of 4 weeks before discarding as negative. These slants were inspected daily during the first week and twice weekly during the next three weeks for growth[40]. Growth on two slants or growth on one medium with presence of hyphal elements in 10% KOH preparation was regarded as significant fungal growth.

Identification of filamentous fungi was done by preparing Lacto Phenol Cotton Blue mount and studying the morphology of hyphae and conidial arrangement. In difficult, ambiguous cases where sporulation was inadequate, Riddle's slide culture technique was performed[23].

In case of yeasts, identification and speciation was done by Gram's stain morphology, germ tube test, morphology on corn meal agar, and biochemical tests by standard microbiological techniques as recommended by CLSI.

Riddle's slide culture technique

This was used to study the undisturbed morphological details of fungi, particularly the relationship between reproductive structures and mycelium.

Procedure:[23]

1. A round piece of filter paper was placed on the bottom of a sterile Petri dish. A pair of thin glass rods was placed on top of the filter paper to serve as supports for a 3 inch × 1 inch glass microscopic slide.
2. A small 1cm block of SDA previously poured into a Petridish was placed on the surface of the microscopic slide. The block was cut using a sterile scalpel.
3. A small portion of the fungal colony to be studied was inoculated onto three or four places in the margins of the agar block using a straight inoculating wire.
4. A coverslip was gently heated by passing it quickly through the flame of a Bunsen burner and immediately placed directly on the surface of the inoculated agar block.
5. A small amount of water was placed into the bottom of the petri dish to saturate the filter paper.
6. The Petridish was incubated at 30^{o}C for 3-5 days.
7. When a growth visually appeared to mature, the coverslip was gently lifted from the surface of the agar with a pair of forceps taking care not to disrupt the mycelium adhering to the bottom of the coverslip.

8. The coverslip was placed on a small drop of LPCB on a second glass slide. Likewise, the mycelium adhering to the surface of the original glass slide after the block was removed also was stained with LPCB and a fresh coverslip was overlaid.
9. The characteristic shape and arrangement of spores was observed microscopically.

All fungal growths were preserved by suspending a small inoculum of spores or conidia in sterile distilled water and kept in sterile cryo vials at room temperature[40].

SENSITIVITY TESTING OF ISOLATES

ANTIMICROBIAL SENSITIVITY FOR BACTERIAL ISOLATES:

Bacterial isolates were subjected to antibiotic sensitivity by the Kirby-Bauer's Disc Diffusion technique on Mueller Hinton agar plates as recommended by CLSI Peptone water culture of the bacterial isolates corresponding to 0.5 McFarland's turbidity was used as inoculum. The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swab dipped into the inoculum[56].

Commercial Hi-Media Antibiotic discs were used. Maximum six antibiotic discs were used for each 9cm diameter plate. These plates were incubated at 37^{o}C for 16–18 hours in ambient air. The diameters of zones of inhibition were interpreted according to CLSI standards for each organism.

Media and discs were tested for quality control using standard strains. The following standard strains were used

- *Staphylococcus aureus*-ATCC 25923
- *Escherichia coli*-ATCC 25922
- *Pseudomonas aeruginosa*-ATCC 27853

The antibiotic discs used for bacterial isolates were:

Antimicrobial agent	Inhibition zone in mm		
	Resistant	Intermediate	Sensitive
Oxacillin 1 mcg	≤ 10	11 – 12	≥ 13
Ciprofloxacin 5mcg	≤ 15	16 – 20	≥ 21
Norfloxacin 10mcg	≤ 12	13– 16	≥ 17
Chloramphenicol 30mcg	≤ 17	18 – 20	≥ 21
Amikacin 30mcg	≤ 14	15 – 16	≥ 17
Cefazolin 30mcg	≤ 14	15 – 17	≥ 18
Gentamicin 30mcg	≤ 12	13 – 14	≥ 15
Vancomycin 30 mcg	-	-	≥ 15

CLSI 2006

MINIMUM INHIBITORY CONCENTRATION (MIC) FOR DETECTING METHICILLIN RESISTANCE:[16]

MIC was performed for Oxacillin by broth microdilution method for *Staphylococcus aureus* isolates to detect Methicillin Resistant *Staphylococus aureus* (MRSA).

The test was performed using Mueller Hinton broth with 2% Sodium chloride in a microtitre plate. The bacterial suspension adjusted to 0.5

McFarlands turbidity was further diluted ten times to give a final concentration of 5×10^5 CFU/ ml in each well.

The range of concentration of Oxacillin used was 0.125µg/ml – 32µg/ml. After inoculation, the microtitre plates were incubated in ambient air at 35^0C for 24 hours. The drug controls and the growth controls were included in each test.

Interpretation

The MIC value is the lowest concentration of Oxacillin that completely inhibits visible growth of the test organism. Growth in the wells with Oxacillin dilutions should be compared with the growth in the control wells for determining the end point.

ANTI FUNGAL SUSCEPTIBILITY TESTS

The antifungal susceptibility testing was done by two methods[64]

- Disc diffusion method
- Broth microdilution method

The Clinical and Laboratory Standards Institute (CLSI) subcommittee on Antifungal Susceptibility Tests has developed a reproducible procedure for antifungal susceptibility testing of filamentous fungi by a broth microdilution format the M-38A document. Recently, an agar diffusion method has been developed for testing filamentous fungi by disc diffusion methodology.

Inoculum preparation

Mould stock inoculum suspensions were prepared from fresh mature (7 day old) cultures grown on Potato dextrose agar following CLSI guidelines. A conidial suspension was prepared by flooding each slant with sterile distilled water. To reduce the hydrophobicity of the conidia and to aid with the formation of uniform conidial suspension of *Aspergillus* species, Tween 80 was added to the sterile distilled water.

The resulting suspension was permitted to stand for 5 minutes to allow large particles to settle down. The suspension was then adjusted spectrophotometrically at 530 nm to the optical density range of 0.09-0.11 for *Aspergillus* species to get an inoculum size of 1.6×10^6 CFU/ ml. The same inoculum was used for both methods[64].

Disc diffusion method

Disc Diffusion test was performed on Mueller-Hinton agar plates supplemented with 2% Glucose and 0.5µg/ml of Methylene Blue[90].

The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swab dipped into the inoculum suspension. The plate was allowed to dry for 20 minutes. Using a pair of flame sterilized forceps the antifungal discs were applied onto the surface of the inoculated plate. The plates were incubated at 35^0C for 48 hours.The plates were read at 24hrs and 48hrs[29].

The following commercial Hi-Media antifungal discs were used

Amphotericin B 100 units

Itraconazole 10µg

Fluconazole 10µg

The following standard strains were tested each time to ensure quality control:

Aspergillus flavus ATCC 204304

Aspergillus fumigatus ATCC 204305

Interpretation

Zone diameters were measured to the nearest whole millimeter at the point where there was prominent reduction of growth[29].

Broth microdilution method

The test was performed in a 96 well microtitre plate using standard RPMI1640 medium. MIC range of antifungal agents used were

- Amphotericin B : 0.03 - 16µg/ml
- Fluconazole : 2 – 256µg/ml

Both these compounds were prepared as 10 mg/ml stock solutions.

Amphotericin B was dissolved in 100% DiMethyl SulphOxide (DMSO)

Fluconazole was dissolved in sterile distilled water[64].

To calculate the volume of compounds to be added to medium from stock solution the following formula was used:

$$V1 \times N1 = V2 \times N2$$

V1 = Volume of stock solution to be added to medium

N1 = Stock compound concentration (10mg/ml)

V2 = Volume of test medium (200 µl)

N2 = Compound concentration to be achieved

To cite an example:

Calculation of V1 for the compound Amphotericin B of concentration 16µg/ml will be

$$V1 \times N1 = V2 \times N2$$

$$V1 \times 1000 \text{ (µg/ml)} = 200 \text{ µl} \times 16 \text{ µg/ml}$$

Thus V1 was calculated for each drug dilution and added to 10 wells.

Each test had:

One medium control (only medium),

One growth control (medium + culture),

One DMSO control (in case of Amphotericin B, DMSO + fungal spores)

Each test was performed in duplicate.

After inoculation, microdilution plates were incubated in ambient air at 35^{o}C and examined for MIC determination at 48 hrs.

Interpretation of MIC

By visual examination, MIC was defined as the lowest drug concentration that showed 100% growth inhibition compared to the growth control well[15].

RESULTS

In this study, a total of 140 cases of corneal ulcers were studied. 107 cases were culture positive, in which 6 cases had mixed infections, thus bringing the isolates to 113.

The cases were analysed under the following parameters:

The age and sex distribution of corneal ulcers were analysed. 77.5% cases were found to be in the 10 – 60 years age group and 26% of corneal ulcer cases were in the 51 – 60 years age group. Extremes of age group (<10 and > 71) showed a low prevalence.

A high prevalence of infectious corneal ulcer cases was seen among males, contributing to 62.6% of cases.

The urban rural distribution of cases showed higher prevalence of corneal ulcers in rural population accounting for 65.4%

Numerous predisposing factors have been implicated in the development of corneal ulcers, of which trauma alone contributed to 50.5% of the cases. Trauma with history of prior treatment with antibiotics / antifungals /

steroids contributed to additional 24% cases. 4.7% cases were found to have diabetes mellitus.

In analysing the contribution of different trauma factors in infectious corneal ulcer, trauma with vegetable matter like paddy, leaf, wood and husk were implicated in 55% cases.

Majority of the aetiological agents were fungi (71%), followed by bacteria (23%). Mixed bacterial and fungal growth was observed in 5.6% of the cases.

Among the fungal isolates, 52 out of 82 (63.2%) cases were due to *Aspergillus* spp. The next common agent was *Penicillium* spp, followed by *Fusarium* spp, *Aureobasidium* spp, *Acremonium* spp and *Candida albicans*.

Staphylococcus aureus was the most common bacterial isolate, accounting for 42%, followed by *Pseudomonas aeruginosa*(39%). *Acinetobacter* spp was isolated in two cases (7%), *Staph.epidermidis, Klebsiella* spp, *Escherichia coli* and *Streptococcus viridans* contributed to 3%

The sensitivity pattern of bacterial isolates revealed 93.5% sensitivity to Amikacin, 67.7% to Chloramphenicol, 61.3% to Norfloxacin ,45.2 % to Cefazolin and 36.7% to Gentamicin.

Four *Staphylococcus aureus* isolates exhibited MIC>2µg/ml for Oxacillin. All these four *MRSA* isolates were sensitive to Vancomycin.

Antifungal sensitivity pattern of fungal isolates by disc diffusion test showed that 30%(3) *Aspergillus fumigatus* isolates, 40%(4) *Aspergillus flavus* isolates, 50%(5) *Aspergillus niger* isolates and 60%(3) *Penicillium* isolates were sensitive to Amphotericin-B. Itraconazole was sensitive in 80%(8) of *Aspergillus fumigatus* isolates, 90% (9) *Aspergillus flavus* isolates, 100%(10) *Aspergillus niger* isolates and 80%(4) *Penicillium* isolates. All the fungal isolates tested were resistant to Fluconazole.

MIC of >2µg/ml (resistance range) of Amphotericin-B was observed in 17 out of 30 (57%) *Aspergillus* isolates by Broth microdilution method. 1 out of 4 *Penicillium* isolates exhibited MIC>2µg/ml.Out of 35 isolates, 29(83%) exhibited MIC >100µg/ml(Resistance range) for Fluconazole.

10% Potassium hydroxide mount preparation used as a screening test for rapid diagnosis of infectious corneal ulcer showed 95% sensitivity and 96.5% specificity .Gram stain preparation showed 86% sensitivity and 98% specificity.

DISCUSSION

Infectious corneal ulceration is a sight threatening condition with significant ocular morbidity requiring prompt treatment. To minimize complications, timely antimicrobial treatment must be initiated on the basis of clinical and microbiological evaluation.

There have been numerous studies both in India and abroad on infectious corneal ulceration in the past 20 years. In all these studies it has been observed that there is changing spectrum of ,agents involved and predisposing factors in different geographical regions.

The present study showed the following results. Out of 140 corneal ulcers studied in detail, 113 aetiological agents were isolated from 107 cases which accounted for 76.4% culture positivity. (Table-1)

This was similar to the study of Geeta K V, et al, 2002, which revealed 78% culture positivity. Bharathi M J, et al, 2002 and Khanal B, et al, 2005, have reported 70% and 67.8% culture positivity in their studies respectively.[31,7,45]

In this study, cases were distributed between children less than ten years to elderly more than seventy years of age group. (113), 77.6% of cases were found to be in 10 – 60 years age group. Children and elderly showed a prevalence rate of 7.5%. (Table-2)

The most common age group affected was 51 – 60 years. This observation correlates well with the study of Bharathi M J, et al, 2003 , who reported higher prevalence among patients aged more than 50 years[7]. The study of Chander J, et al,1994, also showed a higher prevalence of infectious corneal ulcer in 51 – 60 years age group[10].

There was a male preponderance constituting two third of the total study population.p = 0.005 significant. (Table-3)

Similar findings were observed in the study of Chowdhary, et al, 2005, which revealed higher prevalence (68%), among males[13]. Basak samar K, et al, 2005 and Lixen xie, et al, 2006, also reported male preponderance in their studies.[4,55] In contrast, Kotigadde subbannayya, et al, 1992, from Karnataka reported higher incidence (27%), in females than (19%) males.[48]

The rural and urban distribution of corneal ulcer patients in this study revealed higher prevalence of infectious corneal ulcers (65.4%), in people living in rural areas. p = 0.01 significant. (Table-5)

This was similar to the study of Basak samar K, et al, 2005, in which

78.5% of of patients were from rural areas[4]. The studies of Bharathi M J, et al, 2003 and Chander J, et al, 1994, also showed higher prevalence of infectious corneal ulcers in patients from rural background[6,10].

In this study, ocular trauma was the most common predisposing factor. A definite history of antecedent corneal injury was recorded in 74.7% of the patients p = 0.001, significant (Table-6), which was in agreement with the studies of Gopinathan, et al, 2002 and Basak samar K, et al, 2005. In their studies, history of ocular trauma was noted in 54.5% and 83% of patients respectively.[33,4] The studies conducted in abroad by Norina T J, et al, 2008 and Laspinal F, et al, 2004, also revealed history of ocular trauma in 62% and 50% of their patients.[68,49]

In studying the different agents of trauma such as paddy dust, wood, dust etc., 44 cases (55%) were associated with history of injury by vegetable matter. (Table-7)

This correlates with the study of Basak samar K, et al, 2005, according to which 59.6% patients had corneal injury with vegetative matter.[4]

The above observations clearly show that in developing countries where agricultural work is more common, vegetative material induced ocular trauma is the major cause of infectious corneal ulceration.

In this study, 9% of cases gave history of prior antibiotic / antifungal /

topical steroid use, whereas history of trauma and prior treatment was present in 25% of cases (Table-6).

This is similar to the study of Gugnani, et al, who reported that, topical application of steroid or broad spectrum antibiotics alone did not play an important role in the aetiology of infectious corneal ulcers[36].

In the present study, 5% of patients were found to be diabetic. In the study of Basak samar K, et al, 2005, 7.6% of patients had Diabetes mellitus[4] (Table-6).

According to this study, aetiological agents were isolated in 107 (76.4%) samples. Of these 107 culture positive samples 76 (71%) had pure fungal growth, 25 (23%) had pure bacterial growth and 6 (5.6%) had mixed bacterial and fungal growth (Table-4). Fungi were the most common agents ($p = 0.001$ significant) isolated in infectious corneal ulcer in this study (Table – 8).

These observations were similar to the study of Basak samar K, et al, 2005, which revealed 62.7% of pure fungal growth, 22.7% of pure bacterial growth[4]. As per the study of Khanal B, et al, 2005, conducted in Nepal, 47.8% of culture positive samples showed pure fungal growth, 34% showed pure bacterial growth and 18.2% had mixed microbial growth[45].

In contrast the study conducted by Norina T J, et al, 2008, in Malaysia revealed 79.3% of pure bacterial infection, 13.8% of pure fungal infection and

2% of mixed infection[68]. Laspina F et al., 2004, in Paraguay, have reported 51% of pure bacterial infection and 26% of pure fungal infection in infectious corneal ulcers[49].

The above observations clearly show that fungal corneal ulcer is more common in developing tropical countries like India, whereas bacterial corneal ulcers predominated in other countries.

In the present study, 5.6% of samples showed mixed bacterial and fungal growth (Table-4). This correlates with the study of Leck, et a,l 2002, which revealed 5.5% of mixed bacterial and fungal growth[50]. Bharathi M J, et al, 2007, reported 2.5% of mixed microbial growth in their study[5].

Among the fungal agents isolated in this study, 52 (63.2%) were Aspergillus species followed by Penicillium species 13 (16%) and Fusarium species 8 (10%). The remainnig 9 (11%) isolates were Aureobasidium sp, Acremonium sp and Candida albicans (Table-9&10). It is evident from our study that Aspergillus species (52, 63%) was by far the commonest filamentous fungi causing corneal ulcer.

The dominant role of Aspergillus species in infectious corneal ulcer has been reported in the studies of Basak samar K, et al, 2005 and Khanal B, et al, 2005. In their studies, the commonest fungal pathogen was Aspergillus species followed by Fusarium species[4, 45].

In the studies of Lixen Xie ,et al, 2006 and Prashant Garg, et al, 2000, Fusarium species was found to be the most common fungi isolated . In the present study, Fusarium species was isolated only in 10% of samples next to Aspergillus spp. and Penicillium spp.[55,77]. This may be explained by differences in climate and the natural environment.

Aureobasidium species was isolated in 5% of samples in the present study. In the study of Khanal B, et al, 2005, Aureobasidium species was isolated in 12.3% of samples[45].

In the present study, Acremonium species was isolated in 4% of the samples which was closer to the study of Chander J, et al, 1994, where Acremonium species accounted for 6.6% of the isolates[10].

Among the bacterial isolates, Staphylococcus aureus was the most common pathogen isolated in 13 patients accounting for 42%, followed by Pseudomonas aeruginosa, 12 isolates, 39% (Table-11).

This was similar to the study of Basak samar.K, et al, 2005, in which Staphylococcus aureus, followed by Pseudomonas aeruginosa were the most common bacterial pathogens isolated[4]. In the studies of Khanal B, et al, 2005 and Kotigadde subbannayya, et al, 1992, also Staphylococcus aureus was the predominant bacteria isolated[45,48].

The Antibiogram performed for all the bacterial isolates in this study by

Kirby-Bauer Disc Diffusion method showed 93.5% sensitivity to Amikacin, 67.7% to Chloramphenicol, 61.3% to Norfloxacin, 51.6% to Ciprofloxacin, 45% to Cefazolin, 36.7% to Gentamicin (Table-12).

Amikacin was found to be the most effective antibiotic covering 93.3% of isolates in the present study. This observation was similar to the studies of Chien-Fan-Fong et al 2007 and Chalita.M.R, et al, 2004, where Amikacin was found to be effective against 93% - 95% of isolates[12,9]. In the study of Savitiri Sharma, et al, 1999, 71.7% isolates were sensitive to Chloramphenicol, 67.7% to Norfloxacin, 69 % to Ciprofloxacin and 70.6% to Gentamicin[88].

The commonest bacteria isolated in this study, Staphylococcus aureus, exhibited 100% sensitivity to Amikacin, 77% sensitivity to Chloramphenicol. 31% of Staphylococcus aureus isolates were found to be Methicillin resistant (Table-12&13). All Methicillin resistant Staphylococcus aureus (MRSA) strains were sensitive to Vancomycin.

These results were closer to the study of Sotozono C, et al, 2002, where out of 30 MRSA isolated from ocular infection, 12 were isolated from corneal ulcer[96].

Pseudomonas aeruginosa, the second most common bacterial isolate in this study showed a sensitivity pattern of 100% to Ciprofloxacin and Norfloxacin (Table-12).This correlates well with the study of Chein-Fan-Fong, et al, 2007, in which Ciprofloxacin was effective against 99.2% of Pseudomonas keratitis isolates[12].

In the present study , Antifungal susceptibility was performed on 35 isolates for Amphotericin B and Fluconazole both by Disc Diffusion method and Broth microdilution method (CLSI guidelines).It was observed that 30% of Asp. fumigatus, 40% of Asp.flavus and 50% of Asp. niger isolates tested were sensitive to Amphotericin-B by Disc diffusion method. In Broth micro dilution test, MIC < 2µg/ml was observed in 20% of Asp.fumigatus, 50% of Asp. flavus and 60% of Asp.niger isolates. 60% of Penicillium isolates tested were found to be sensitive to Amphotericin-B by Disc diffusion method, 80% isolates exhibited MIC < 2µg/ml (Table-14&15).

All the fungal isolates tested (100%), were resistant to Fluconazole by Disc diffusion method and 11% isolates, exhibited MIC < 100 µg/ml by Broth micodilution test (Table-14&16).

The results obtained reveal good correlation between the Broth microdilution method and Disc diffusion method for Antifungal susceptibility testing of filamentous fungi. Espinel-Ingroff, et al, 2007, have reported good correlation of inhibition zone diameter with MIC for filamentous fungi[29].

Antifungal susceptibility to Itraconazole on 30 Aspergillus isolates by Disc diffusion method revealed 90% sensitivity (Table-14). This correlates with the study of Sun Xuguang, et al, 2007, in which 92% of Aspergillus species isolated from cornea were sensitive to Itraconazole[100].

In evaluating the screening tests for rapid diagnosis of aetiological agents in infectious corneal ulcers, 10% Potassium hydroxide (KOH) mount and Gram's stain examination of the corneal scrapings were analysed.

10% KOH mount examination showed a sensitivity of 95% and specificity of 96.5% (Table-17). This correlates with the study of Vajpayee.R.B, et al, 1993, which revealed 94.3% sensitivity of 10% KOH mount examination[108]. Bharathi M J, et al, 2007, reported 99% sensitivity and 1.5 % false positive rate of KOH wet mount preparation. The false positive rate of KOH smear in the present study was 1.4%[5].

In the present study, the sensitivity of Gram's stain was 86% and specificity was 98% in the diagnosis of bacterial agents. Bharathi M J, et al, 2007, reported 100% sensitivity of Gram's stain examination. Sharma, et al, 2002, reported 41% sensitivity and 87% specificity of Gram's stain preparation[5,92].

The results of the present study showed the vital role of 10% KOH mount and Gram's stain examination in the diagnosis of infectious corneal ulceration. Although culturing of microbial pathogens is considered to be the gold standard, direct microscopic evaluation of smears provide immediate information about the aetiological agents and aid in early initiation of antimicrobial therapy.

SUMMARY

- Totally 140 infectious corneal ulcers were studied in detail. Aetiological agents were isolated in 107 (76.4%) cases.
- Majority of the isolates were fungal agents (72.6%), belonging to the Genus *Aspergillus* (63.2%) ,followed by *Penicillium* spp. (16%) and *Fusarium* spp.(10%).
- Bacterial corneal ulcers were less common (27.4%). The predominant bacterial pathogen isolated was *Staphylococcus aureus*, followed by *Pseudomonas aeruginosa*.
- Male preponderance was observed (63%) in this study.
- The age group most commonly affected was between 51 and 60 years.
- Incidence of infectious corneal ulcer was more in rural population than urban population.
- Trauma with vegetative matter was found to be the most common predisposing factor (51%) in the development of infectious corneal ulcers.
- 90% of fungal isolates were sensitive to Itraconazole. 43% of isolates were sensitive to Amphotericin B. All fungal isolates were resistant to Fluconazole by Disc diffusion method.
- 52% fungal isolates exhibited MIC in the resistance range (>2µg/ml) for Amphotericin B and 83% fungal isolates exhibited MIC in the resistance range (>100µg/ml) for Fluconazole by Broth microdilution method.

- There was a good correlation between antifungal susceptibility results obtained by Broth microdilution method and Disc diffusion method.
- Majority of the Bacterial isolates were sensitive to Amikacin.
- The 4 *MRSA* strains isolated were sensitive to Vancomycin.
- The sensitivity of 10% KOH mount (95%) was comparatively higher than Gram stain examination (86%).
- 10% KOH mount and Gram's stain procedures were found to be highly sensitive as rapid screening tests for diagnosing corneal ulcers of fungal and bacterial aetiology respectively.

CONCLUSION

In conclusion, the infectious corneal ulceration was predominant in adult males of rural background, with vegetative matter induced ocular trauma as the major predisposing factor. A simple KOH preparation was highly beneficial as a rapid screening test for diagnosis. Fungal corneal ulcers were more common than bacterial ulcers. *Aspergillus* spp. and *Staphylococcus aureus* were the most common fungus and bacteria causing corneal ulcers respectively. From the present study, the vital role of microbiological evaluation in the management of infectious corneal ulcer is clearly evident, since the clinical features alone are not adequate to confirm infection. It is important to create awareness among people especially from rural background with regard to trauma as a major predisposing factor for corneal ulcers. Precise identification of the causative organisms and timely institution of appropriate antimicrobial therapy based on the prevailing sensitivity pattern of the fungal and bacterial isolates could save the eye from this preventable cause of blindness.

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PROFORMA

S.No. : Date: I.P./O.P.No.:

Name : Age/Sex: Occupation:

Address :

Presenting Complaints: Duration:

History of

- Trauma
- Prior Treatment
- Contact Lens wear
- Similar recurrent complaints
- Diabetes
- Hypertension

Clinical Examination

- General Physical Examination

  Built / Nourishment Pulse BP

- Ocular examination

  Eye lid

  Conjunctiva

  Cornea - Position, Size of Ulcer, Margins. Slough,

  Satellite Lesions, Corneal sensation.

  Anterior Segment Examination - Pupils, Hypopyon

  Vision

Investigation

- Staining with 2% fluorescein
- Syringing of nasolacrimal duct
- Blood sugar

Microbiological Investigations

- Direct Microscopy

Screening tests

Gram's stain

10% KOH Mount

- Culture

Bacterial culture

Mac Conkey Agar

5% Sheep blood agar

Brain heart infusion broth

Fungal culture

Sabouraud's Dextrose Agar

Antibiotic sensitivity test

Mueller-Hinton Agar

Antifungal sensitivity test

Mueller-Hinton Agar

RPMI 1640 Broth

APPENDIX

A. STAINS AND REAGENTS

I. 10% KOH

Potassium hydroxide l0g

Glycerol – l0ml

Distilled water - 80ml

II. Gram staining

- Methyl violet (2%) – l0g Methyl violet in l00ml absolute alcohol in 1litre of distilled water (primary stain)
- Grams Iodine – l0g Iodine in 20g KI (fixative)
- Acetone – Decolourising agent
- Carbol fuchsin 1% – Secondary stain

III. Lactophenol cotton blue stain

Lactic acid	-	20 ml
Phenol	-	20ml
Cotton blue (dye)	-	0.5g
Glycerol	-	40ml
Distilled water	-	20ml.

Media used

1. **Mac Conkey agar**

Peptone		- 20g
Sodium taurocholate	-	5g
Distilled Water	-	1 ltr
Agar		- 20g

2% neutral red in 50% ethanol - 3.5ml

10% lactose solution - l00ml

Dissolve peptone and taurocholate in water by heating. Add agar and dissolve it in steamer. Adjust pH to 7.5. Add lactose and neutral red shake well and mix. Heat in free steam (100°C) for 1 hour, then autoclave at 115°C for 15 minutes.

2. **Blood agar (5% sheep blood agar)**

Peptone - l0g

Nacl - 5g

Distilled water - 1 Ltr

Agar - 10g

Dissolve ingredients in distilled water by boiling, and add 5% sheep blood (sterile) at 55°C adjust pH to 7.4.

3. Brain heart infusion broth

Sodium citrate - 1g

Sodium chloride - 4g

Sodium phosphate - 5g

Dextrose - 10g

Peptone - 10gm

Brain Heart Infusion

Brain infusion broth - 250ml

Heart infusion broth - 750ml

Sodium polyanethol sulphonate - 0. 25g

Obtain ox brain and heart. Remove all fat from heart and cut into small pieces

and grind. Add distilled water three times and keep it at 4°C overnight.

From the brain remove the meninges fully and then weigh. Add distilled water and mash by using hand .Keep cooler overnight. Next morning boil the brain and heart separately for 30 minutes. Then filter through cotton layer. Measure each broth separately. Mix both infusions and the remaining ingredients. Dissolve well and adjust the pH to 7.4-7.6.

Autoclave at 121°C for 15 minutes. Filter through filter paper and distribute in screw capped bottles in 50 to 100 ml amounts. Autoclave again at 115°C for 10 minutes.

4. Sabouraud's dextrose agar

Dextrose	-	40g
Peptone	-	l0g
Agar	-	20g
Distilled water	-	l000ml

pH = 5.5

5. **Mueller- Hinton Agar**

Beef infusion	-	300ml
Caesein hydrolysate	-	17.5g
Starch	-	1.5g
Agar	-	10g
Distilled water	-	1ltr

pH = 7.4

Sterilise by autoclaving at 121^0C for 20 mins

6. RPMI 1640 Broth

Commercially purchased RPMI 1640 media was dissolved in 1000ml of sterile distilled water. The pH was adjusted to 7.0

The medium was sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns.

MEDIA REQUIRED FOR BIOCHEMICAL iDENTIFICATION OF BACTERIA

1.Oxidase Reagent

Tetra methyl p-phenelene diamine dihyrochloride-

1% aqueous solution.

2.Catalase

3% hydrogen peroxide

3.Indole test

Kovac's reagent

Amyl or isoamyl alcohol	-	150ml
Para dimethyl amino benzaldehyde	-	10g
Concentrated hydrochloric acid	-	50ml

Dissolve the aldehyde in the alcohol and slowly add the acid. Prepare in small quantities and store in the refrigerator. Shake gently before use.

4.Christensen's Urease test medium

Peptone	-	1g
Sodium chloride	-	5g
Dipotassium hydrogen phosphate	-	2g
Phenol red	-	6ml
Agar	-	20g
Distilled water	-	1 ltr
10% sterile solution of glucose	-	l0ml
Sterile 20% urea solution	-	l00ml

Sterilize the glucose and urea solutions by filtration. Prepare the basal medium without glucose and urea, adjust to pH 6.8-6.9 and sterilize by autoclaving in a flask at 121°C for 30min. Cool to about 50°C, add the glucose & urea, and tube the medium as slopes.

5.Simmon's Citrate Medium

Koser's medium	-	1 ltr
Agar	-	20g
Bromothymol blue 0.2%	-	40ml

Dispense, autoclave' at 121°C for 15 min and allow to set as slopes

6.Triple Sugar Iron medium

Beef Extract	-	3g
Yeast extract	-	3g
Peptone	-	20g

Glucose	-	1g
Lactose	-	l0g
Sucrose	-	l0g
Ferric citrate	-	0.3g
Sodium chloride	-	5g
Sodum thiosulphate	-	0.3g
Agar	-	12g
Phenol red 0.2% solution	-	l2ml
Distilled water	-	1 ltr

Heat to dissolve the solids, add the indicator solution, mix and tube. Sterilize at 121°C for 15 min and cool to form slopes with deep butts.

7.Glucose phosphate broth

Peptone	-	5g
Dipotassium hydrogen phosphate	-	5g
Water	-	1 ltr
Glucose 10% solution	-	50ml

Dissolve the peptone and phosphate and adjust the pH to 7.6. Filter dispense in 5ml amounts and sterilize at 121°c for 15min. Sterilize the glucose solution by filtration and add 0.25ml to each tube.

Methyl Red Reagent

Methyl Red	-	10mg
Ethyl alcohol	-	30ml
Distilled water	-	20ml

Voges Proskauer Reagent

Reagent A: Alpha naphthol	-	5g
Ethyl alcohol	-	100ml
Reagent B: Potassium hydroxide	-	40g
Distilled water	-	100ml

8.Peptone water fermentation test medium.

To the basal medium of peptone water, add sterilised sugars of 1% indicator bromothymol blue with Durham's tube.

Basal medium peptone water

Sugar solutions:

Sugar	-	1ml,
Dislilled water	-	l00ml

pH = 7.6.

9.Mannitol motility medium

Agar	-	5g
Peptone	-	1g
Potassium nitrate	-	1g
Mannitol	-	2g
Phenol red indicator		
Distilled water	-	l000ml

pH = 7.2