A STUDY ON ISOLATION AND CHARACTERISATION OF FUNGAL AGENTS CAUSING CORNEAL ULCER IN A TERTIARY CARE HOSPITAL

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled "**A STUDY ON ISOLATION AND CHARACTERISATION OF FUNGAL AGENTS CAUSING CORNEAL ULCER IN A TERTIARY CARE HOSPITAL"** submitted by **Dr.K.R.PANDIARAJ"** to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the requirement for the award of M.D degree Branch– IV (Microbiology) is a bonafide research work carried out by him under direct supervision & guidance.

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CONTENTS

INTRODUCTION

INTRODUCTION

The eye forms an important organ for sensory reception[82]. It is made up of 3 main layers outer fibrous layer,uveal tract and retina. The outer fibrous layer includes sclera and cornea of which corneal layer is the most protective layer to the eye. Any disruption or damage to the corneal layer creates major impact in the vision and cause many visual disturbances. Microscopically the cornea consists of five layers[40,43],

(a) The corneal epithelium with its basement membrane

(b) The Bowman's layer

(c) The Stroma

(d) The Descemet's membrane

(e) The Endothelium

Corneal ulcer is defined as a loss of cornel epithelium with underlying stromal infiltration and suppuration associated with signs of inflammation[29]. Corneal blindness is a major public health problem and infectious keratitis is one of the predominant and preventable cause. Corneal ulcer may be caused by trauma, allergy or infection. Infection may be due to either bacteria, virus,fungus or a parasite. Conditions like trauma, steroid therapy and immunosuppressive states like Diabetes mellitus render

the cornea susceptible to bacterial, fungal, parasitic infections[10]. Any damage to the corneal epithelium pave way for the micro organisms to enter and get into the cornea and produce ulceration and infection thereby leading to visual disturbances. It is a suppurative ulcerative, and life threatening infection of the cornea and sometimes leads to loss of vision.

In South east Asia, according to an estimate 6.5 million people are affected with corneal ulcer and 1.3 million eyes are blind due to corneal ulcer every year[91]. In India 1.5 to 2 million people are affected with corneal ulcer[54]. In Madurai the annual incidence of corneal ulcer is 11.3 per 10,000 population[33].

Studies on microbial infection of the eye are increasing in respect with the mortality and morbidity due to ocular emergencies[130]. The presence of fungi in the corneal ulcer seems to vary not only from place to place but also with relation to the their occupation [44,134] . Particularly people working with the decaying vegetation like mouldy hay in agriculture were more prone to develop infectious corneal ulcer[63] . Minor trauma to corneal epithelium leads to direct implantation of fungal spores leading to corneal ulcer[73,99.]

As clinical diagnosis can't provide a clear picture of causative organism, microbiological evaluation is very important in the diagnosis and treatment of corneal ulcer. Among the causative micro organisms of

corneal ulcer, the incidence of fungal agents causing corneal ulcer has been increasing in recent years[77,130]. So there is a need for a study to know the recent and change in trends in the commonest fungus causative for this corneal ulcer and the changes in the antifungal susceptibility pattern in recent years. The fungal isolates commonly associated with infectious corneal ulceration are Aspergillus species, Penicillium species and Fusarium species[7,49].

Direct microscopic evaluation of smears provide immediate information about the causative organism and is helpful in starting antimicrobial therapy in a short course of time[49]. 10% Potassium hydroxide mount, Gram stain, Fungal culture and Lactophenol cotton blue mount are the commonly employed procedures employed for diagnosis of fungal diseases[30.]

Early diagnosis help in treatment of corneal ulcer and thereby it helps to reduce the occurrence of blindness. As resistance patterns to antifungal drugs continue to shift, sensitivity testing play an important role in appropriate management of individual cases based on susceptibility characteristics, to decrease the complications, spreading and also for community surveillance[86]. Broth micro dilution method is employed to study the sensitivity pattern of fungal agents to antifungal drugs.

So, considering the importance of corneal ulceration and its impact on vision, the present study is conducted to identify the predisposing factors of corneal ulcers, the etiological fungal agents and their susceptibility profiles in patients attending a tertiary care ophthalmic hospital in Madurai.

AIMS &
OBJECTIVES

AIM AND OBJECTIVES

1. To isolate and identify the fungal agents causing corneal ulcer in a tertiary care hospital.

2. To study the co relation between rapid identification by KOH mount and conventional culture method.

3. To study the sensitivity pattern of fungal isolates by susceptibilty to antifungal drugs by broth microdilution method.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Corneal ulceration is the leading cause of ocular morbidity and blindness all over the world[143]. In 1801 Antonia scarpa wrote the first textbook on eye diseases[57]. The first fungus causing corneal ulcer was documented in 1879 by Leber[120,139]. That case of fungal kerartitis was caused by Aspergillus glucus. Mycotic corneal ulcer started reportedly in many parts of the world including India. The aetiological agents involved in infectious corneal ulcer can be classified as Bacterial, Fungal, Viral, Protozoal[29]. The Fungal agents causing corneal ulcer are[50,137]

Hyaline hypomycetes:

(a) Aspergillus species

(b) Acremonium species

(c) Penicillium species

(d) Fusarium species

(e) Pseudallescheria species

Phaeoid hypomycetes:

(a) Aureobasidium pullulans

(b) Alternaria species

(c) Bipolaris species

(d) Curvularia species

(e) Cladosporium species

Yeast like fungi:

(a) Candida albicans

(b) Candida krusei

(c) Candida tropicalis

EPIDEMIOLOGY

Fungal keratitis is a major blinding eye disease in Asia[123]. The problem of keratomycosis in India is more acute because of the humid environment, poverty, illiteracy and ignorance. As per the review article by M. Srinivasan[110], one report from south India found that 44% of all central corneal ulcers are caused by fungi. This high prevalence of fungal pathogens in south India is significantly greater than that found in similar studies in Nepal (17%), Bangladesh (36%), Ghana (37.6%) and South Florida(35%). In temperate climates, such as Britain and the northern United States, the incidence of fungal keratitis remains very low. The incidence of fungal keratitis varies according to geographical location and ranges from 2% keratitis cases in New York to 35% in Florida[123].

Vinay Agarwal et al in a review article in the year 1994 stated that fungal corneal ulcers were very common and represent 30 to 40% of all cases of culture positive infectious keratitis in South India[1].

M.Srinivasan et al studied 434 corneal ulcer patients in 1994 over a period of three months at Aravind eye hospital Madurai (South India) and reported fungal etiology in 32% of cases and bacterial etiology in 32.3% of cases[122].

In 1991, Gupta et al studied on conjunctival flora of 62 patients of corneal ulcer and identified 15 patients (25%) had fungal invasions.

In 1992, a study conducted in Karnataka showed Aspergillus fumigatus as the commonest fungus causing corneal ulcer.

In 1995 Povis et al conducted a study in Jhamnagar and reported Fusarium species as the commonest fungus causing corneal ulcer[96].

Verenkar M P et al in 1998 study reported 12.5% of corneal ulcer are caused by Penicillium species[141].

Liesegang and Foster in 1999[46] conducted study in South Florida in six hundred and sixty three patients and identified 20.1% are due to fungal agents and the most common fungal agent was documented as Fusarium species and the next common agent is Aspergillus species[65].

MJ Bharathi et al conducted a study in 1999-2001 in Tirunelveli (Tamilnadu) to identify the specific microbial pathogens responsible for corneal ulceration in South India[12]. In the 18 months period, 1618 patients

with corneal ulcerations were evaluated. Corneal cultures were found to be positive in 1126(69.59%) patients. Of the 1618 patients, 566(34.98%) had bacterial growth, 522(32.26%) had fungal growth, 30(1.85%) had mixed bacterial and fungal growth[12].

Another study conducted in Trichy by Philip and Thomas showed Fusarium as the commonest fungus causing corneal ulcer[94].

In Madurai, Savithri Sharma et al conducted a study and showed high prevalence of Fusarium species among the isolates causing corneal ulcer[113].

Usha Gopinathan et al analyzed 5897 suspected cases of microbial keratitis between 1991 and 2001 at L.V Prasad Eye Institute, Hyderabad, India. They reported fungal etiology in 38.2% of patients and bacterial etiology in 51.9% of patients[42].

Samar K Basak et al studied 1198 patients with suppurative keratitis over a period of three years from 2001 to 2003 at Disha Eye hospital, Barrackpore in West Bengal and found Cultures were positive in 811(67.7%) patients. Among these culture positive cases 509(62.7%) patients had pure fungal infections[9].

Namrata kumara et al in 2002 study showed 7.89%of cases of corneal ulcer are caused by Penicillium species[84].

Anil Kumar et al evaluated 200 cases of suspected microbial keratitis from 2003 to 2005 and reported fungal etiology in 22% of patients with microbial keratitis[61].

A prospective study of corneal ulcer was conducted in Sari, between May 2004 and March 2005 by Tahereh Shokohi. Fungi were identified as the principal etiologic agents of corneal ulceration in 7(31.8%) patients out of 22 patients[115].

Vijaya S.Rajamane reported 33.78% of fungal keratitis in study conducted from Jan 2005 to Dec 2005, at Shri Chatrapati Shivaji Maharaj General hospital Solapur,Maharashtra[100].

In 2005, Chowdary et al did study on spectrum of Fungal keratitis in North India covering the epidemiology and laboratory results of fungus causing corneal ulcers[17].

In a study done by Reema Nath et al in Assam medical college from 2007 to 2009, fungal etiology was obtained in 60.6% of corneal ulcer patients[85].

Suman Saha et al conducted study to determine the epidemiological characteristics of fungal keratitis in an urban population of West Bengal and identify the specific pathogenic organisms in 2008. Of the 289 patients of microbial keratitis included in the study, 110 patients (38.06%) were diagnosed with fungal keratitis (10% KOH mount positive)[112].

The fungal etiology was reported in 39.12% of cases in retrospective study from 2007 to 2011 by Bandyopadhyay S et al, at a tertiary care hospital in Kolkata[8].

In a study done by Upadhyay et al in Nepal[139], the commonest fungal agent causing corneal ulcer was identified as Aspergillus species and the second common fungus was isolated as Fusarium species.

A retrospective chart review of all patients who had a positive fungal culture from corneal scrapings and diagnosis of fungal keratitis presenting from1996 to 2004 at the Royal Victorian Eye and Ear Hospital was performed by Prashant Bhartiya et al. *Candida albicans* (37.2%) was the most common fungal isolate followed by *Aspergillus* spp (17.1%) and *Fusarium* spp (14.3%).

Dan He et al studied 174 patients with clinically presumed fungal keratitis with corneal ulceration at the China –Japan Union Hospital of Jilin University from 2004 to 2009[23]. A total of 160 patients (92%) were diagnosed with fungal infection by either KOH wet mount or microbiologic culture. Fungal cultures were positive in 73.6% patients. *Fusarium* (48.2%) was the most commonly isolated fungus followed by *Aspergillus* spp (18.7%).

PATHOGENESIS

Fungi can invade the eye in the following manners

1. By direct invasion of the external eye and results in fungal conjunctivitis fungal keratitis and fungal infection of the lacrimal passage.

2. Extension from infected neighbouring structures as in fungal dermatitis nasopharyngitis and sinusitis.

3. Entry into the interior of the eye by perforating wounds, operating wounds postoperatively.

4. Suppression of antifungal biological safety mechanism in the conjunctiva.

The fungi are unable to penetrate intact corneal epithelium hence any trauma particularly, organic matter facilitate penetration of fugal inoculums into corneal stroma. The fungal hyphae invade from corneal ulcer to stroma. Coagulation necrosis associated with loss of keratocytes and oedematous changes of collagen fibres occur. Satellite lesions are formed around main site of involvement. Late in course of disease process, hyphae may be seen in Descemet's membrane, encased in dense neutrophilic exudates of hypopyon.

They multiply and cause tissue necrosis and elicit inflammatory reaction. They can penetrate the intact Descemets membrane and gain access into the anterior chamber or the posterior chamber resulting in the

exogenous endophthalmitis. Mycotoxins and proteolytic enzymes of fungi augment the tissue damage[138].

CLINICAL FEATURES:

It frequently manifests within 24-36 hours following trauma. The early biomicroscopic features consist of fine or coarse granular infiltrates within the epithelium and anterior stroma, with minimal cellular reaction. The epithelium has dry, rough texture and dirty gray white color. The epithelium may be elevated and intact or occasionally it may be ulcerated. Mild inflammation may contribute to the irregular edges of the feathery infiltrates. There may be multifocal suppurative micro abscess or satellite lesions. Occasionally, pigmentation in the ulcer bed is seen in demateacious fungal keratitis. The lack of marked stromal infiltration may permit direct visualization of pigment and delicate, feathery, branching hyphae with surrounding stromal infiltrate. A white ring in the cornea is frequently present and presumably represents a toxic fungal diffusate and interaction of fungal antigen and host antibody. Mild iritis tends to occur early, but an endothelial plaque and hypopyon generally takes several days to develop. Dense fibrinous material adheres to the endothelium and collects in the anterior chamber and over the surface of the iris. With advanced disease, the entire cornea becomes homogeneously yellow white and can resemble any severe microbial keratitis. Stromal ulceration necrosis can lead to perforation and endophthalmitis.

Yeast keratitis occurs in a different clinical setting. These patients have pre-existing ocular inflammatory disease or severe alterations in ocular structures. Trauma alone is rarely the initiating event. Yeast keratitis occurs in association with systemic diseases, such Sjogren's syndrome, erythema multiforme, IgA deficiency, cell mediated immune deficiency, human immunodeficiency virus infection and endocrinopathies. Yeast keratitis causes a small discrete, sharply demarcated dense yellow- white stromal suppuration that lacks the delicate features of filamentous organisms. Yeast keratitis resembles a gram positive bacterial keratitis such as Staphylococcus aureus or Streptococcus pneumonia keratitis.

The patients generally present with the complaints of pain, watering, redness, photophobia, diminished vision usually presented unilaterally and vision blurred. On examination there may be conjuctival chemosis, congestion, purulent discharge, hypopyon and stromal infiltration. In addition, the presenting clinical features that are specific to fungal ulcers include a greyish white infiltration with feathery margins, rough texture and raised borders with endothelial plaques, satellite lesions and folds in Descemet's membrane. The surrounding corneal stroma is oedematous. The presence of pigmented infiltrate may be an important diagnostic clue for phaeoid fungi.

DIAGNOSIS:

SPECIMEN COLLECTION:

Corneal scraping are collected under strict aseptic precautions by an ophthalmologist using sterile No.15 Bard Parker blade[3] after instillation of a local anaesthetics like 2% lignocaine hydrochloride from leading edge of the ulcer[1].

MICROSCOPY OF SMEARS

1.**10%Potassium hydroxide (KOH) Mount**[7,132]:

Corneal scrapings were placed in a glass slide with 10% KOH to see the fungal elements.

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 reports become available, thus proving to be sight saving[16]. In 2007 Bharathi *et al* concluded that KOH smear has a greater diagnostic value in the diagnosis of fungal keratitis[13].

2. **Gram Stain**[7,132]:

Smears are prepared from corneal scraping and Gram staining was done to observe the bacteria and yeast like cells.

Bharathi *et al* in 2006 reported 100% sensitivity of Gram stain procedure in the diagnosis[13].

3. **Calcofluor white stain**[7,132]:

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. To the corneal scraping in a slide, 1 drop of 0.1% calcofluor white with 0.1% Evans blue and 1 drop 10% KOH are added. A coverslip is placed over the specimen and examined under fluorescent microscope. The morphology of smaller fungal elements was better appreciated in calcofluor white mount.

Chandar *et al* in 1993 reported that fungi could be detected in corneal tissue by calcofluor white staining in 95.2% of patients, where KOH mount and culture were positive in 89.6% of patients.

FUNGAL CULTURE[7,132]:

Microbial culture is considered to be the gold standard in the detection of causative organism of corneal ulcer. Inoculated Sabourauds dextrose agar slant were incubated aerobically at 25˚c over a period of 6 weeks. Culture was checked every day during first week and twice weekly thereafter. Fungal isolates are identified by their colony characteristics, morphology in obverse and reverse, microscopic morphology in lactophenol cotton blue mount and slide culture.

Lactophenol Cotton Blue mount[132]**:**

Lactophenol Cotton Blue mount was used to observe the hyphal and conidial arrangement and conclude the fungal growth with culture[136].

Thomas *et al* in1991 and Sharma *et al* in 1998 documented the correlation of macroscopic morphology with microscopic findings in LPCB mount[135]. Kompa *et al* in 1999 used LPCB mount as a sensitive marker in diagnosis.

Slide Culture[132]:

The slide culture was performed using isolates. The slide culture is used to study undisturbed morphology details particularly relationship between reproductive structures like conidia, conidiophores and hyphae[59,128]. Adhesive Method for Microscopic Examination of Fungi in Culture were used to improve the identification[109].

ANTIFUNGAL SUSCEPTIBILITY TESTING:

Antifungal susceptibility testing is done by agar based, broth based and colorimetric methods.

METHODS:

1.**Agar based methods**

a. Agar dilution

b. Disk diffusion

c. E test

2**. Broth based methods**

a. Broth macrodilution

b. Broth microdilution

3. **Colorimetric method**

AGAR DILUTION METHOD[133]

The drug of various concentrations added to the Nutrient agar slope and inoculum suspension was added. The MIC (Minimal Inhibitory Concentration) was determined as the lowest concentration of the antifungal drug preventing the growth of macroscopically visible colonies on drug containing plates, when there was visible growth on the drug free control plates. For MIC determination, the following range of drug concentrations were

Amphotericin B : 0.0313-16µg/ml

Itraconazole : 0.0313-16 µg/ml

Fluconazole : 0.125-64 µg/ml

DISK DIFFUSION METHOD[106]:

This method is useful in vitro testing of antifungal agent against standard inoculation of fungal pathogen. Disk diffusion method will provide sensitivity pattern of particular fungal pathogen by comparing with the standard zone size. Reference method for disk diffusion susceptibility testing of filamentous fungi, approved guideline M 51-A is followed[106]

E-TEST METHOD:

E- test is a patented commercial method for determination of MIC. In this method calibrated plastic strip impregnated with a concentration gradient of antifungal agent placed over the agar surface and zone of inhibition corresponding to concentration gradient is noted. Inoue T *et al*

documented E- test in choosing appropriate agents to treat fungal keratitis[47].

BROTH MACRODILUTION METHOD[31]:

Broth macrodilution was performed in sterile 6 ml polystyrene tubes with a final volume of 1 ml two times the required concentrations of the drug and the conidial suspension were prepared by two fold serial dilutions.

BROTH MICRODILUTION METHOD[31]:

The clinical and laboratory standards institute (CLSI) subcommittee on Antifungal susceptibility tests has been developed a reproducible procedure for antifungal susceptibility testing of filamentous fungi by a broth microdilution format M 38-A2 document for filamentous fungi[105]. It recommends the use of RPMI-1640 medium with glutamine without bicarbonate supplemented with 0.2% glucose and buffered to a pH of 7.0 with 0.165 mol/L MOPS (3-N- morpholinopropane sulfonic acid). Inoculum preparation of conidial or sporangiospore suspensions must be adjusted using a spectrophotometer in a range of $0.4x10^4$ to $5x10^4$ CFU/ml to get the most reproducible MIC data. A small drop of Tween 20 as wetting agent added to facilitate the preparation of Aspergillus inoculum. Standard two fold serial dilutions across the concentration range to be tested are made. Good agreement between results obtained by broth microdilution and broth macrodilution methods for moulds has been documented.

COLORIMETRIC METHOD:

Tetrazolium salts can penetrate rapidly with intact cells and directly with subcellular membrane with dehydrogenase activity, where they are converted to coloured formazan derivative that can be measured spectrophotometrically at 550nm. Tellier et al in 1992 showed 56% positivity in his study[97]. Pfaller and Barry in 1994 used Alamar blue, anovel colorimetric indicator that changes colour from blue to red[93].

Other diagnostic methods:

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

Impression cytology and confocal microscopy are other diagnostic tools which are not used routinely. Confocal microscopy is a new and non invasive procedure in which four dimensional view of internal structures are possible at cellular level. Zhonghua *et al* 1999 documented 31 out of 43 patients with fungal keratitis with 96.9% positive rate by confocal microscopy[143].

Detection of fungal metabolites by gas liquid chromatography[48]

Flow cytometry:

Flow cytometry gives the results within 6 hours. Ramani and Chaturvedi in 2000 reported the antifungal susceptibility of fungal pathogen by flow cytometry[101]

SEROLOGY

a. Detection of antibody

b. Detection of antigen

MOLECULAR DIAGNOSIS

Polymerase chain reaction

SEROLOGY:

1. Detection of antibody:

The antibody production depends on host factor, causative fungus and type of infections. Coleman and kaufman in 1972 found precipitin in 82% of proven cases of fungal corneal ulcer[21]. Solid phase radio immunoassay has been developed for measurement of antibody which was used in the study by Marier *et al* in 1999[75]. Monoclonal antibody based ELISA was also developed for the detection of antibody levels of fungus causing corneal ulcer[15].

2. **Detection of antigen**:

The serological test for detection of antigens are of limited value in early stages of infection, in patient with impaired immunity or immune response is not sufficient to raise significant level of antibodies. Latex particle agglutination test for detection of antigen were used. Radio immunoassay (RIA) shows 70-80% sensitivity in study conducted by Talbot *et al* in 1987[127]. Sabetta *et al* in 1985 demonstrate antigen by competitive enzyme immune assay (EIA) in five of six immuno compromised cases with invasive fungal infection[111].

MOLECULAR DIAGNOSIS:

Polymerase chain reaction

Polymerase chain reaction amplification can be used to detect the presence of as few as 10 organisms per 100ml volume of clinical specimen. PCR used to detect segment of fungus specific DNA coding for cytochrome $P450L_1A_1$, chitin synthase gene, 18S RNA gene. Corneal scrapings are processed for DNA extraction which is amplified by fungal specific primers of internal transcribed spacer region 1 (ITS 1). The products are sequenced and analysed by single standard conformation polymorphism (SSCP) for species identification.

Manish kumar *et al* in 2005 has reported, the sensitive and rapid polymerase chain reaction based diagnosis of mycotic keratitis through single standard confirmation polymorphism in their study[74].

Detection and Identification of fungal pathogen by PCR and by ITC2 and ribosomal DNA typing in ocular infection by Consuelo Ferrer *et al* in 2001.

TREATMENT OF CORNEAL ULCER:

ANTIFUNGAL AGENTS IN FUNGAL CORNEAL ULCER[34]:

The commonly used antifungal drugs for corneal ulcers are

1. Polyene antibiotics - Amphotericin B,Natamycin
2. Azoles; Triazoles-

   Flucanazole, Voriconazole, Itraconazole, Posaconazole, Ravuconazole
3. Miscellaneous- Flucytosine, Echinocandin

Natamycin 5% suspension, Amphotericin B is used routinely in the treatment of corneal ulcer. The Azoles and Flucytosine are generally used an alternative agents in advanced ulcers[94]. Oral Fluconazole and Itraconazole have good intraocular penetration with few adverse effects compared to other azoles[34]. Newer agents like as triazoles (Posaconazole,

Ravuconazole), Echinocandins, Sodarin derivaties and the Nikkomycins will improve the treatment of fungal corneal ulcer[36].

Surgical treatment of corneal ulcer:

Frequent cornea debridement with a spatula is helpful which debulks fungal organisms and epithelium and enhances penetration of the topical antifungal agents[34]. Although mainstay of initial management of severe keratitis remains aggressive antimicrobial therapy, the role of timely surgical intervention in the form of therapeutic keratoplasty[49] should be considered in patients with severe end stage diseases. The timing of surgery was critical. The surgery should be performed within 4 weeks of presentation. Therapeutic keratoplasty may effectively treat severe refractory infectious corneal ulcers[114].

Considering the previous studies it is clear that the fungal agents play a major role in causing corneal ulcer and it is necessary to study the recent changes in the distribution of the commonly isolated fungal agents causing corneal ulcer and to study the changes in the susceptibility pattern of the fungal agents to antifungal drugs. So this study helps in isolation and identification of the common fungal agent causing corneal ulcer in patients attending ophthalmic OPD in a tertiary care hospital. Gram stain, rapid identification by KOH mount and conventional fungal culture by Sabouraud dextrose agar slant culture and Lactophenol cotton blue mount

are some of the tests to identify the fungus causing corneal ulcer. Co relation between rapid identification by KOH and conventional culture method is done to find the reliability of both the tests. This study is also used to study the sensitivity pattern of fungal isolates to antifungal drugs by broth micro dilution method thereby helps in appropriate treatment of fungal corneal ulcer.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present study was conducted in patients attending in OP and admitted in Ophthalmic ward at Government Rajaji Hospital, Madurai and specimens were processed in Institute of Microbiology, Madurai Medical College. Ethical approval has been obtained from the Institutional Ethical Committee, Madurai Medical College. The specimens were collected after getting written informed consent from the patients.

Study type **:** Prospective study

Study Period **:** July2018 to June 2019

Study population **:** The study population consists of patients attending in Ophthalmology OP and Ophthalmic ward at Government Rajaji Hospital, Madurai.

Sample size **:** 100

Specimen **:** Corneal scraping

Inclusion Criteria:

Patients with signs and symptoms of corneal ulcer such as pain redness, itching, watering of the eye, dimness of vision and photophobia. All age groups and both sexes are included in this study.

Exclusion criteria:

Patients who are in immunocompromised state and Antenatal mothers are excluded from this study.

Collection of specimen[1]:

Written consent from the participants (or) their guardians included in of the study was obtained after providing full explanation of the current study in their local language. All the data collected were kept confidential. Standard operating procedures were followed doing sample collection[3].Specimens were taken from patients of corneal ulcer and follow-up patients with corneal ulcer. Informed consent was obtained from the patients and data were collected as per proforma. Corneal scrapings were collected for investigations.

1. Patient was made to lie down comfortably on a couch
2. The affected eye was cleaned with sterile saline using sterile swabs.
3. Sterile 2% Xylocaine was applied to the eye taking care not to apply too much of it as it may inhibit the growth of the organism.
4. Care was taken to see that the eyelids did not contaminate the specimens. Eye speculum was used whenever necessary.

5. Patients were given relevant instructions regarding position and restriction of eyeball movement during the scraping procedure.

6. No.15 Bard Parker blades were used to scrap the ulcer. A new sterile blade was used for each patient.

7. Materials were obtained from leading edge and base of each ulcer. Scrapings were taken and processed as follows.

a. Specimen was applied to two sterile microscope slide for 10% KOH mount and Grams stain.

b. Specimens were inoculated into two Sabouraud dextrose agar slants with antibiotics (Gentamicin) without Cycloheximide.

SPECIMEN PROCESSING:

1. 10% POTASSIUM HYDROXIDE MOUNT[7,132]:

The scraping material was transferred onto a clean glass slide and one or two drops of sterile 10% KOH was applied over that and covered with clean coverslip without introducing air bubbles and examined under low and high power objective for the presence of hyphal elements, conidial forms of the fungal isolates. KOH digests proteinacious material and retain the polysaccharide fungal cell wall. The results will be correlated with culture report later.

2. Direct Grams stain[7,132]:

The corneal scraping material was transferred onto a clean glass slide with a drop of sterile normal saline. The smear was made using a sterile bacteriological loop. The smear was allowed to air dry and heat fixed. The prepared smear was stained by Gram stain method and examined under oil immersion objective and observed for presence of polymorphs, mononuclear cells, epithelial cells, bacteria(Gram positive & Gram negative), yeast like cell, if present their nature and relative number were noted. Bacterial pathogen identified and processed.

3. Culture Method [7,132] :

Microbial culture is considered to be the gold standard in the detection of causative organism of corneal ulcers. The Bard Parker blade containing the scraping material was slightly depressed in to the Sabourauds dextrose agar slant medium, so that the specimen was left on the surface. The SDA slopes were incubated at 37^0 C and 25^0 C for 4 weeks.

Fungal isolates were identified by studying the colony morphology on the Sabouraud dextrose agar slope, colony colour, production and arrangement of conidia in preparation stained by Lactophenol cotton blue mount. When identification was difficult due to inadequate sporulation, Riddles slide culture technique was

employed. In the case of yeast identification,it was done by Gram stain morphology, Germ tube test, morphology on Corn-meal agar and biochemical test by standard microbiological techniques.

EXAMINATION OF INOCULATED MEDIA[132]:

The colonies were observed for growth in the Sabouraud dextrose agar and noted the description, if it was inadequate reincubated. The Sabouraud dextrose agar slopes were examined daily during first week and twice a week for next 3 weeks. Failure of growth after 6 weeks was considered as negative for fungal growth and is to be discarded.

LACTOPHENOL COTTON BLUE MOUNT[132]:

The fungal growth was taken from Sabouraud dextrose agar slope with spud and transferred onto the clean glass slide and two to three drops of Lactophenol cotton blue reagent was added over the fungal growth. By using teasing needles the growth was spread over the slide and coverslip was placed without trapping any air bubbles. The morphology of hyphae, conidia were observed under microscope and was correlated with macroscopic features.

RIDDLE'S SLIDE CULTURE METHOD[132]:

This was used to study the undisturbed morphological details of fungi, particularly relationship between reproductive structures like conidia

conidiophores and hyphae. Fungal slide culture was performed in cases with doubtful morphology.

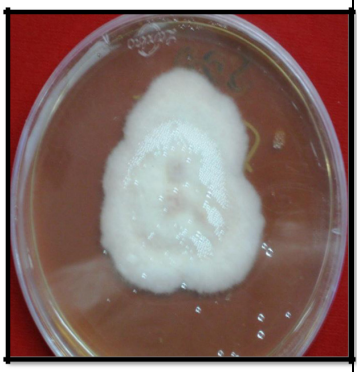
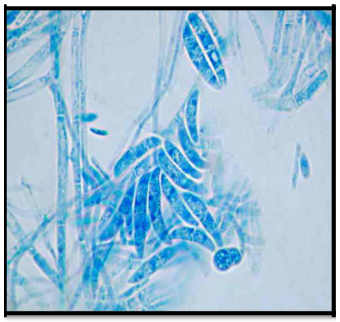
1. A round piece of filter paper was placed on the bottom of a sterile Petri dish. A pair of thin glass rods was placed on top of the filter paper to serve as supports 3 inch x 1 inch glass microscopic slide. 3 to 4 coverslips were placed within the petridish and sterilized as a whole.

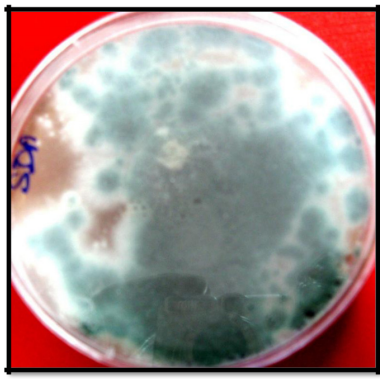
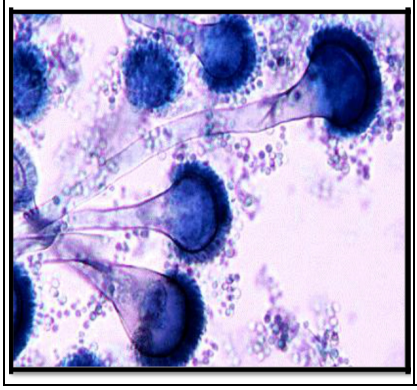
2. 1x1 cm square block of Sabouraud dextrose agar was cut from a petridish by using sterile scalpel and transferred the agar block to the microscope slide.
3. Four sides of the agar block were inoculated with a fungal colony to be studied by using heavy gauge nichrome wire.
4. The agar block was covered with sterile coverslip in the petridish.
5. Moisten the filter paper with sterile water and place the lid on the petridish.
6. The Petridish was incubated at room temperature and examined periodically for growth.
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 disturb the mycelium adhering to the bottom of the coverslip.

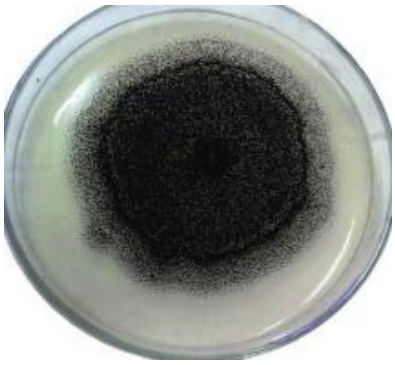
8. The coverslip was placed on a small drop of Lactophenol cotton blue on a second glass slide. Likewise, the mycelium adhering to the surface of the original glass slide after the block removed also was stained with Lactophenol cotton blue and a fresh coverslip was overlaid.
9. The characteristic shape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

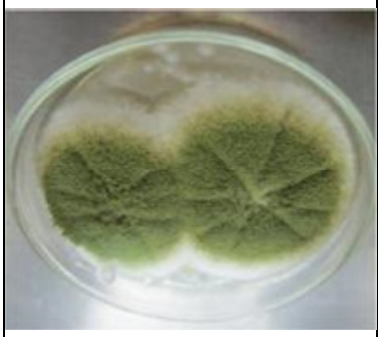
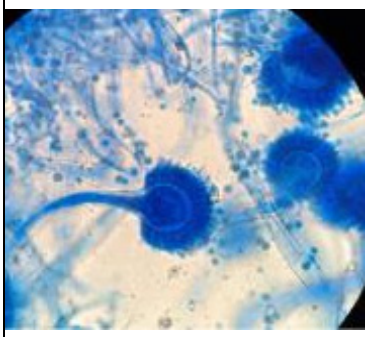
The mycelia which adhere to the glass surface usually show characteristic microscopic appearance which may be lost if needles are used to tease as it happens in the routine Lactophenol cotton blue mounts. The slide culture may also be seen directly by placing under low power of the microscope. The cellophane tape preparation has come into greater use to overcome the obstacles of time consumption and requirement of the extra equipment to preparethe slide culture. A piece of tape is gently laid over a portion of the fungal colony and slowly lifted to remove an area of the colony and placed on a microscope slide with a drop of Lactophenol cotton blue and examined under low power of the microscope. This preparation becomes an instant slide culture, revealing relationship of the various fungal structures.

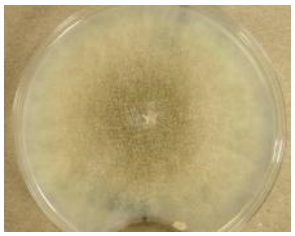
Identification of individual fungi[27]: Fungal isolates from the corneal scrapings were identified based on the characteristic colony morphology on SDA slant and microscopic appearance.

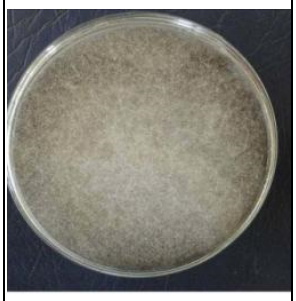
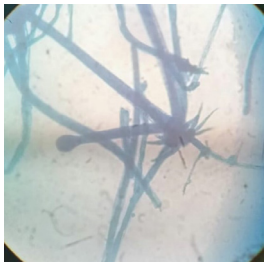
Organism	Colony morphology	Microscopic appearance
Fusarium species  	White and cottony initially and later turns to pink. Reverse is usually light, but may be deeply colored.	Hyphae appears septate Two types of conidia: 1. Sickle shaped macro conidia measuring (2- 6) x (14-80) µm with 3-5 septa. 2.Short simple conidiophores measuring (2- 4) x (4-8) µm with 1-2 septations.

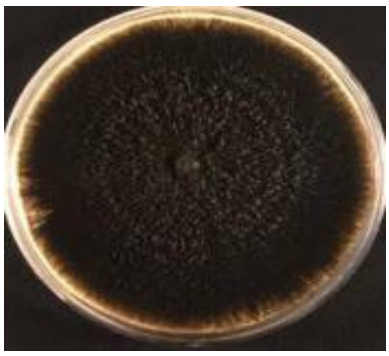
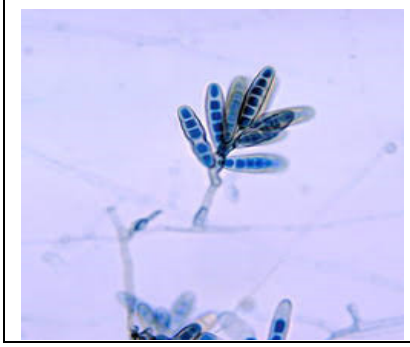
Aspergillus fumigatus  	Appears velvety or powdery initially and later turns to darkish to gray. Reverse is white to tan.	Conidiophores are short and smooth. Phialides are uniseriate, usually only on upper two- thirds of vesicle, parallel to axis of conidiophores.

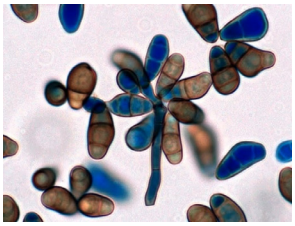
Aspergillus niger  	Woolly, initially white to yellow and later turning to black. Reverse is white to yellow.	Conidiophores are long and smooth. Phialides are biseriate and cover entire vesicle

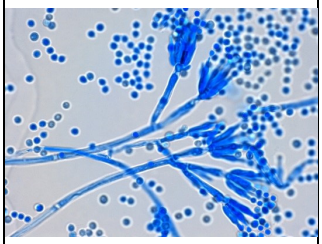
Aspergillus flavus  	Velvety, yellow to green or brown. Reverse is golden to red- brown	Conidiophores are variable in length and rough Phialides are uniseriate and biseriate; cover entire vesicle

***Mucor* species**  	Growth quickly covers agar surface with fluff resembling cotton candy Reverse is white	Hyphae are wide (6-15 µm), non-septate. Sporangiophores are long, often branched and bear terminal round, spore filled sporangia (50-300µm in diameter).

***Rhizopus* species**  	Growth rapidly covers agar surface with dense cotton candy like; colonies are white at first and then gray or yellowish brown. Reverse is white.	Hyphae are broad (6-15µm in diameter) with no or very few septations. The Sporangiophores are long up to 4mm and terminate with a dark, round sporangium (40-350µm in diameter) containing columella and many oval, colorless or brown spores (4-11µm in diameter). It has stolons, rhizoids and usually unbranched sporangiophores.

***Bipolaris* species**  	Surface is grayish brown initially and later becomes black with a matted center and raised grayish periphery. Reverse is dark brown to black.	Hyphae are dark septate. Conidiophores elongate and bend at the point where each conidium is formed. The conidia are brown, cylindrical(6-12x16-35µm), appear thick walled and have 3-5 septations.

***Curvularia* species**  	Colony is dark olive-green to brown or black with a pinkish gray, wooly surface. Reverse is dark.	Hyphae are septate and dark. Conidiophores are simple or branched and bent at points of conidium formation. Conidia are large(8-14) x(21-35) µm, usually contain 4 cells and eventually appear curved due to swelling of a central cell

Penicillium species  	Colonies are blue green with a white border and powdery surface, Reverse is pale yellow.	Septate hyphae with branched conidiophores, with 2 rows of sterigmata bearing chains of spores having brush or boom appearance.

ANTI FUNGAL SUSCEPTIBILITY TESTING:

The National Committee for Clinical laboratory Standards (NCCLS) which describes the standard parameters for testing MIC (Minimum Inhibitory Concentration) of established agents against filamentous fungi[19,20]. Antifungal susceptibility testing is receiving attention with the advent of newer anti-fungal drugs. However susceptibility testing of filamentous fungi is not as advised as susceptibility testing. In vitro susceptibility tests should provide a reliable measure of relative activity of the antifungal agent, correlate with in vivo activity and predict the likely outcome of the therapy, provide a means with which to monitor the development of resistance and predict the therapeutic potentials of newer drugs.

Invitro Susceptibility Testing of fungi is influenced by a number of technical variables such as inoculums size and preparation, medium composition and pH, duration and temperature of incubation and MIC end point determination. In addition there are problems unique to fungi like their slow growth rates and the ability of some of them to grow either as yeasts with blastoconidia or as moulds with variety of conidia depending on pH, temperature and medium composition.

BROTH MICRODILUTION METHOD[105]

1.Growth Medium Preparation[19]:

1. The completely synthetic medium Rosewell Park Memorial Institute – 1640 (RPMI-1640) supplemented with 0.3g of L-glutamate per liter without sodium bicarbonate was used as a growth medium in antifungal susceptibility testing. The medium should be buffered at the pH of 7.0 ± 0.1 at 35˚c.

2. The buffer used was MOPS (3-N-morpholinopropane sulfonic acid) with final concentration of 0.165 mol/L with ph of 7.0 .

3. RPMI 1640 was dissolved in MOPS. The final solution was sterilized by filtration through membrane filter and stored at 4˚c.

4. The same medium was used for the preparation of the drug dilutions.

2.Drug Dilution Preparation[19]:

1. The drug dilutions were prepared following the additive twofold drug dilution scheme described in the NCCLS M38-A method[19].

2. Stock drug solutions were first diluted to 100x the final concentration in100% dimethyl sulfoxide (DMSO) and further diluted 1:50 in 2x medium to obtain the 2x drug concentration. The final drug concentration was 0.125 to32µg/ml for Amphotericin B

and 0.0313 to 16 µg/ml for Itraconazole. Fluconazole was dissolved in sterile distilled water and final drug concentration was made from 2 to 256 µg/ml.

3. These volumes were adjusted according to the total number of tests required. Because there will be 1:2 dilution of the drug when combined with the inoculum, working antifungal solutions were 2 fold more concentrated than the final concentration.

3. Inoculation In RPMI – 1640 Medium[105]:

1. The inoculation was done in sterile 96 - well microtitre plate with flat bottom.
2. Each well was inoculated with 100 µl of the conidial suspension
3. 100µl of the diluted drugs were added correspondingly to each well.
4. The growth control well was inoculated only with the 200 µl of diluted conidial suspension with the growth medium without any antifungal agents.
5. The sterility control well was inoculated with 200 µl of the growth medium alone without any conidium.
6. All microtitre plates were incubated at 35°C for 48 hours without agitation and evaluation was done after four days of incubation.

4. Reading MIC[20]:

The test was read when the growth control shows adequate growth, which is typically 24-48 hours for most moulds, but it could be up to 96 hours.

Read MICs the first day that the growths controls showed the visible growth and then 24 hours later.

Scores were given as follows,

(1) 1. 0 = optically clear

(2) 1+ = slightly hazy

(3) 2+ = prominent reduction in turbidity compared with that of the drug-free growth control

(4) 3+ = slight reduction in turbidity compared with that of the drug-free growth control

(5) 4+ = no reduction in turbidity compared with that of the drug-free growth control.

STATISTICAL ANALYSIS:

A statistical analysis was carried out. The test outcome was observed, recorded and analysed. The data that were analysed was presented in the form of statistical tables, pie charts and histograms in

appropriate places. P values were calculated by Pearson Chi-Square and Fishers exact Chi-square test and found to be <0.05 for the study.

The data were documented and studied in detail. The documented data was further discussed in detail and compared with other similar studies published in reputed scientific journals.

RESULTS

RESULTS

A total of 100 samples are isolated from patients having corneal ulcer from Ophthalmic Op and patients admitted in Ophthalmic ward in Government Rajaji Hospital, Madurai.

TABLE 1

CULTURE POSITIVITY IN THE CORNEAL SCRAPING SAMPLES N=100

Total no. samples collected	No. of Culture positive Samples	Percentage of culture positivity
100	32	32%

Table 1 shows out of 100 samples, 32 samples (32%) are culture positive.

CHART -1

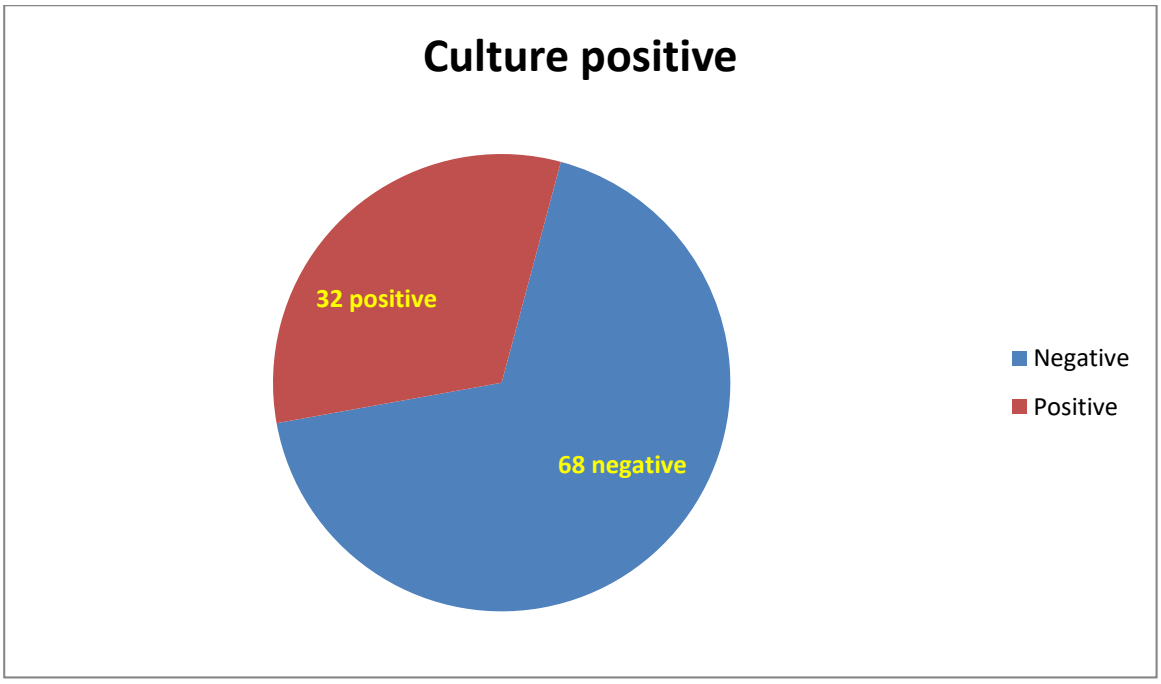


Chart 1 shows out of 100 samples, 32 samples are positive for fungal culture and 68 samples are negative for fungal culture.

TABLE 2

GENDER DISTRIBUTION OF INFECTIOUS CORNEAL ULCER N=100

Gender	Total No. of cases	No. of culture positives	Percentage
Male	62	24	75%
Female	38	8	25%

Table 2 shows male (75%) are more commonly affected by fungal corneal ulcer than female (25%)

CHART-2

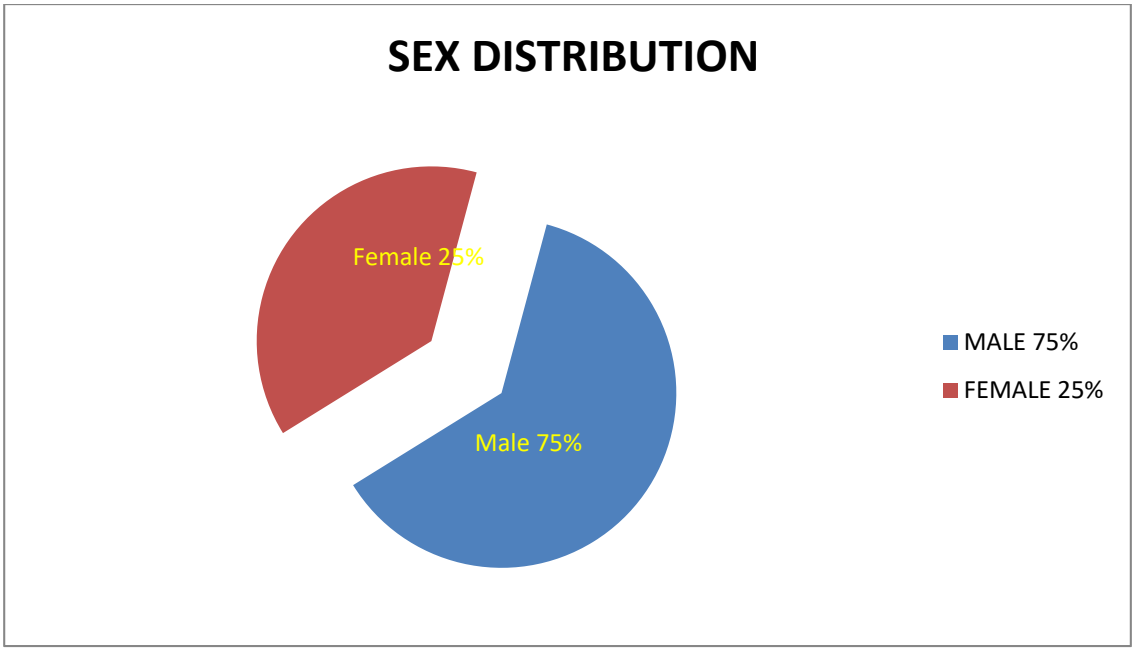


Chart 2 shows males (75%) are more commonly affected by fungal corneal ulcer than female (25%).

TABLE 3

AGE DISTRIBUTION OF INFECTIOUS CORNEAL ULCER N=100

Age (Years)	Total No. Of Cases	No.of culture positive		Percentage of cases on total culture positive (%)
		Males	Females	
10	-	-	-	-
11-20	2	1	0	3.13
21-30	8	3	1	12.5
31-40	16	4	1	15.63
41-50	38	8	3	34.38
51-60	30	6	2	25
>60	6	2	1	9.38
Total	100	24	8	100.0

Table 3 shows age group between 41 to 50 years (34.38%) are more commonly affected by fungal corneal ulcer.

CHART -3

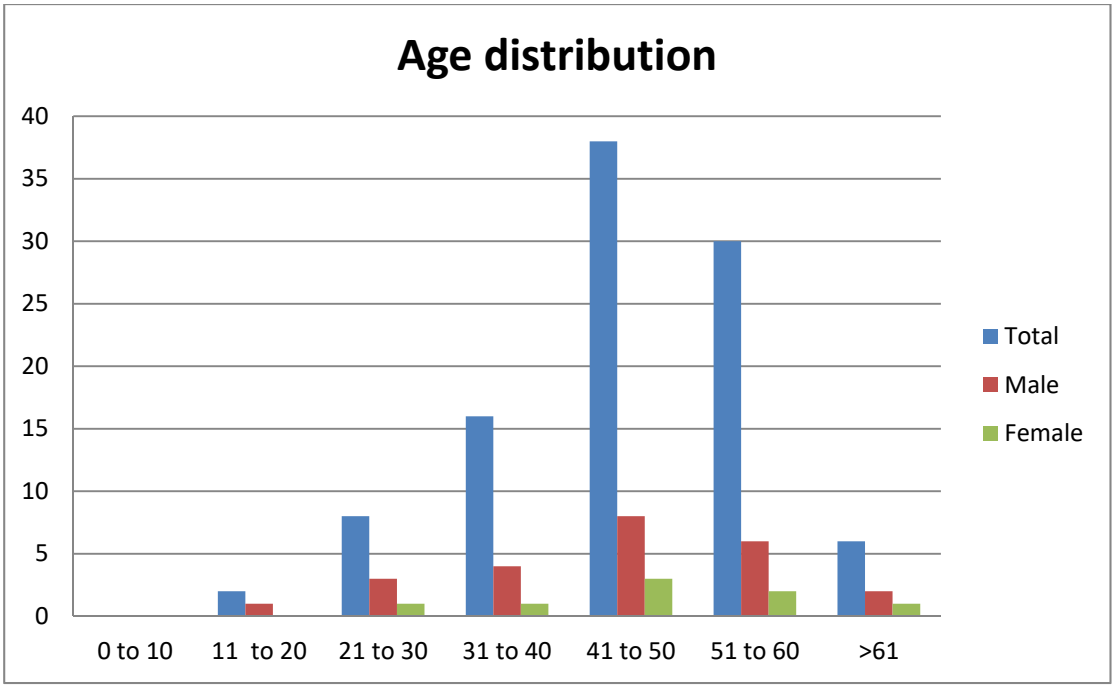


Chart 3 shows majority of population belonging to the age group between 41 and 50years are affected by fungal corneal ulcer

TABLE 4

DISTRIBUTION OF FUNGAL AGENTS CAUSING CORNEAL ULCER

Fungal Agent	Total isolates	No. of isolates		Percentage (%)
		Male	Female	
Aspergillus fumigatus	11	7	4	34.38
Aspergillus flavus	7	5	2	21.88
Aspergillus niger	6	5	1	18.75
Fusarium species	5	4	1	15.63
Penicillium species	2	2	0	6.25
Curvularia species	1	1	0	3.13
Total	32	24	8	100

Table 4 shows majority of the isolates were belonging to Aspergillus species (75%) of which Aspergillus fumigatus accounts for 34.38%, Aspergillus flavus accounts for 21.88% and Aspergillus niger accounts for 18.75% followed by Fusarium species (15.63%) , Penicillium species (6.25%) and Curvularia species (3.13%).

CHART -4

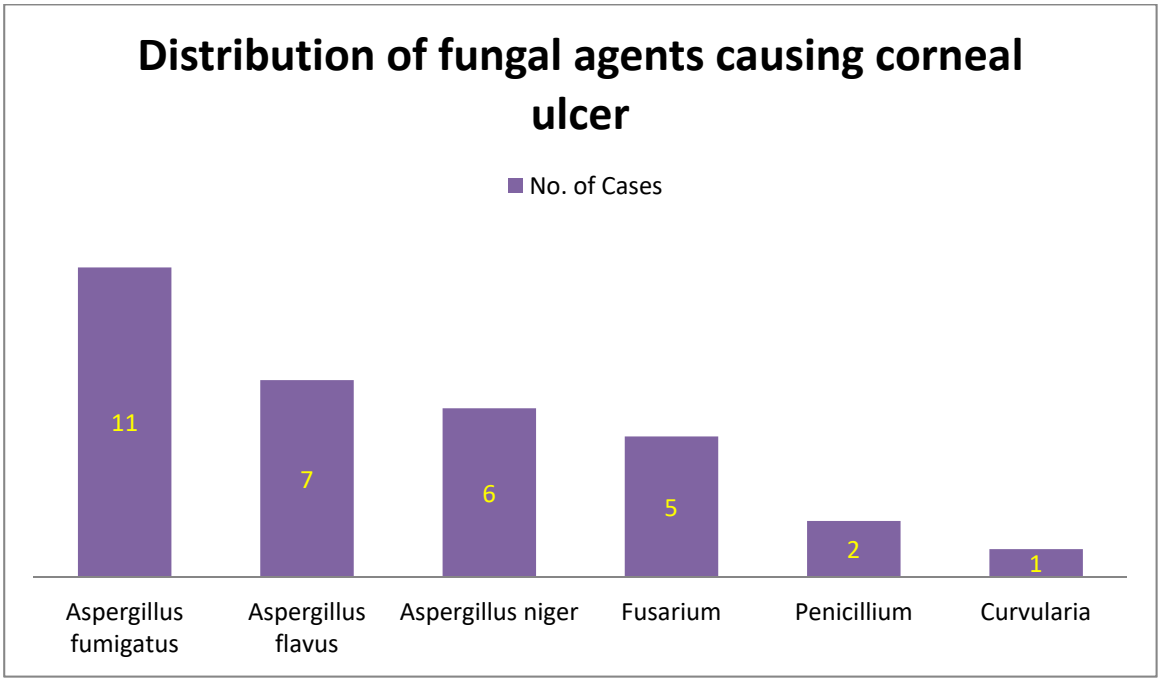


Chart 4 shows majority of fungal corneal ulcers are caused by Aspergillus fumigatus followed by Apergillus flavus, Aspergillus niger, Fusarium, Penicillium and Curvularia species.

TABLE 5

SMEAR POSITIVITY AMONG CORNEAL ISOLATES

Gender	Total No. of specimens	10%KOH positivity
Male	62	25
Female	38	8

Table 5 shows 33 samples are positive for 10% KOH mount : 25 are male and 8 are female.

TABLE 6

CO RELATION BETWEEN 10%KOH AND FUNGAL CULTURE POSITIVITY

10% KOH Mount	Culture		Total
	Positive	Negative	
Positive	31	2	33
Negative	1	66	67
Total	32	68	100

Table 6 shows out of 33 positivity for 10% KOH mount, 31 are positive for culture and 2 are negative for culture . Out of 32 positivity for culture, 31 are positive for 10% KOH mount and 1 is negative for 10% KOH mount. The sensitivity and specificity are as follows

Sensitivity : True Positive / (True Positive + False Negative) = 96.9%

Specificity : True Negative / (True Negative + False Positive) = 97%

TABLE 7

MINIMUM INHIBITORY CONCENTRATION AMPHOTERICIN B BROTH MICRODILUTION METHOD

Organism	0.25µg	0.5µg	1µg	2µg	4µg	8µg	16µg	32µg	64µg
Aspergillus fumigatus	-	3	6	1	1	-	-	-	-
Aspergillus flavus	1	2	3	1	-	-	-	-	-
Aspergillus niger	2	1	3	-	-	-	-	-	-
Fusarium species	-	1	3	1	-	-	-	-	-
Penicilium species	-	-	2	-	-	-	-	-	-
Curvularia species	-	1	-	-	-	-	-	-	-

Table 7 shows 21 samples (80.77%) of Aspergillus species, 4 samples (80%) of Fusarium species, 2 samples (100%) of Penicillium species and 1 sample (100%) of Curvularia species are having MIC less than 2µg/ml and are sensitive to Amphotericin B.

TABLE 8

MINIMUM INHIBITORY CONCENTRATION ITRACONAZOLE BROTH MICRODILUTION METHOD

Organism	0.125 µg	0.25µg	0.5µg	1µg	2µg	4µg	8µg	16µg	32µg
Aspergillus fumigatus	-	-	1	3	1	3	2	1	-
Aspergillus flavus	-	1	2	4	-	-	-	-	-
Aspergillus niger	-	2	3	1	-	-	-	-	-
Fusarium species	-	1	2	-	1	1	-	-	-
Penicillium species	-	-	1	1	-	-	-	-	-
Curvularia species.	-	-	1	-	-	-	-	-	-

Table 8 shows 17 samples (70.83%) of Aspergillus species , 3 samples (60 %) of Fusarium species,2 samples (100%) of Penicillium species and 1 sample (100%) of Curvularia species are having MIC less than 2µg/ml and are sensitive to Itracanazole.

TABLE 9

CO RELATION OF CULTURE POSITIVE CASES AND OCCUPATION

Culture positive	Occupation								
	Farmer	**Coolie**	**Shopkeeper**	**Welder**	**Housewife**	**Contracter**	**Carpenter**	**Land owner**	**Student**
Male	14	2	2	2	-	1	1	1	1
Female	3	2	1	-	2	-	-	-	-
Total	17	4	3	2	2	1	1	1	1

Table 9 shows majority of the fungal corneal ulcer are seen in farm workers (53.12 %)

FIGURE -1

A CASE OF CORNEAL ULCER AND SPECIMEN TAKEN BY SCRAPING

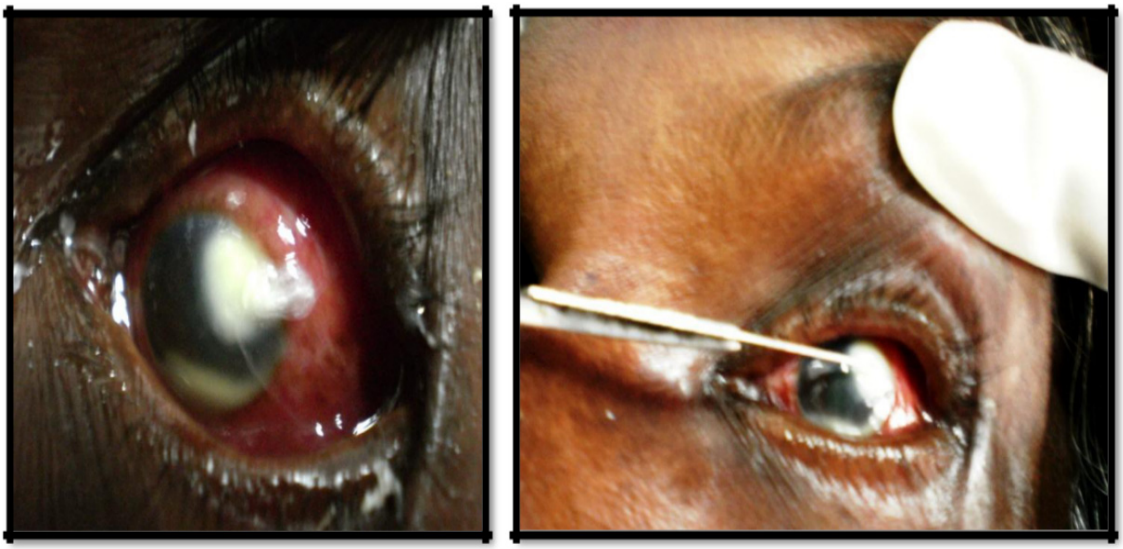


Fig 1 shows taking the sample of a corneal scraping from a patient with corneal ulcer under aseptic precautions

FIGURE -2

10% KOH MOUNT

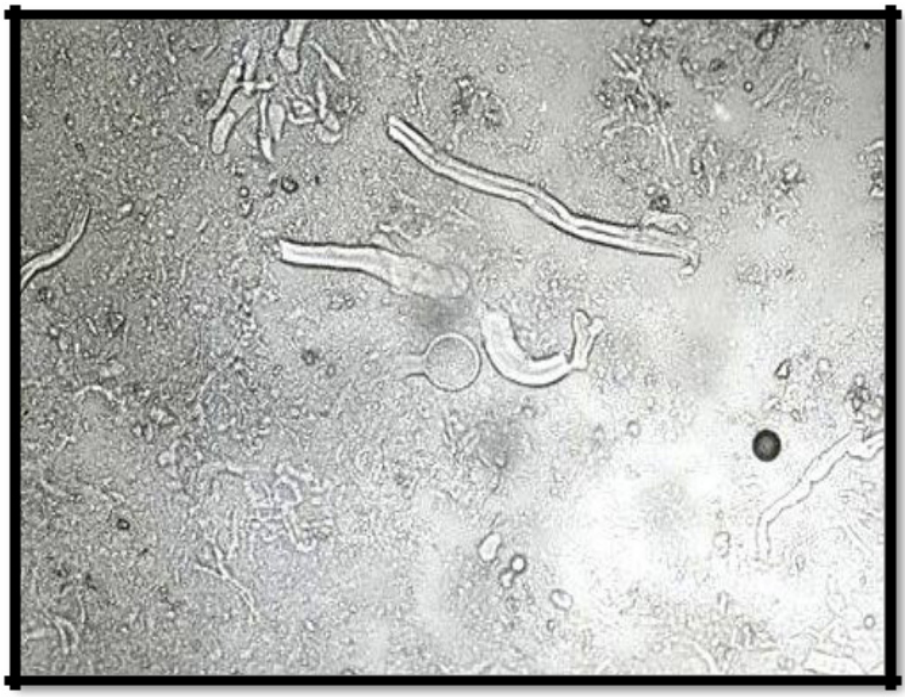


Fig 2 shows 10% KOH mount of a corneal scraping sample showing the hyphal elements suggestive of fungal growth.

FIGURE -3

SLIDE CULTURE

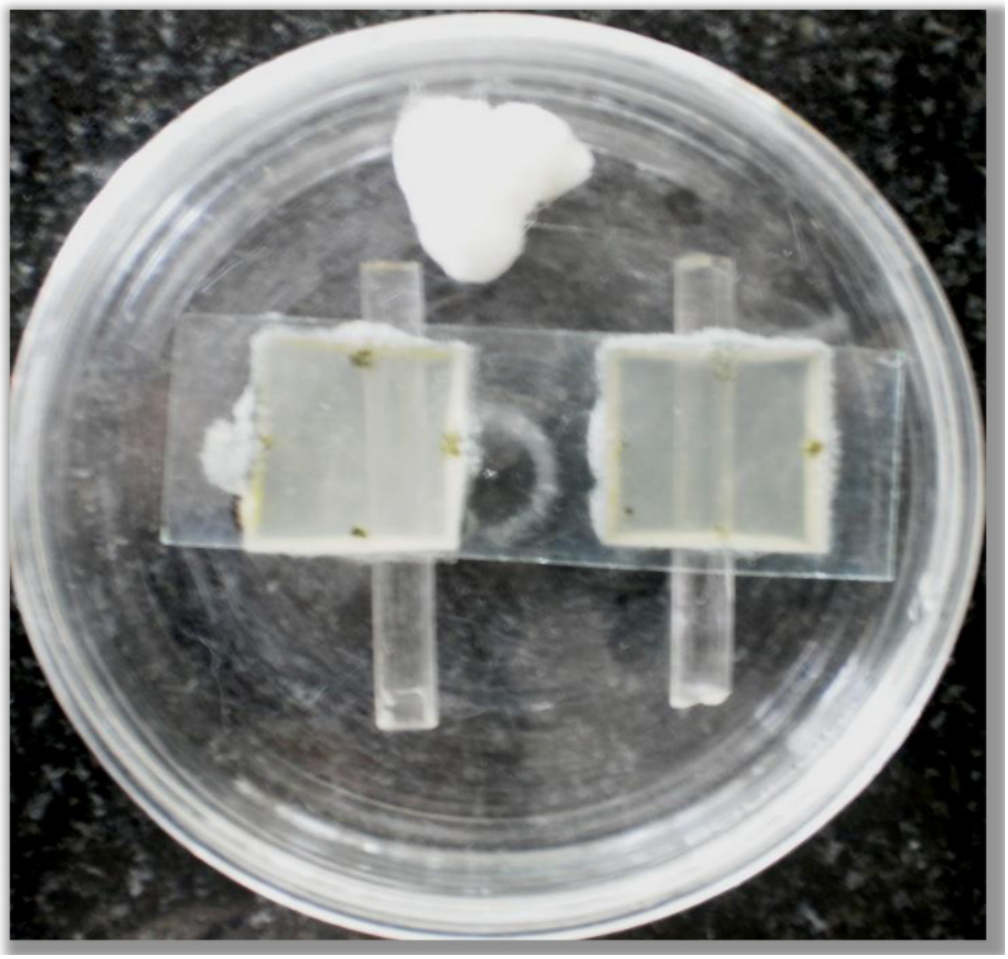


Fig 3 shows slide culture of fungal growth done by Riddles slide culture method

FIGURE -4

ASPERGILLUS NIGER

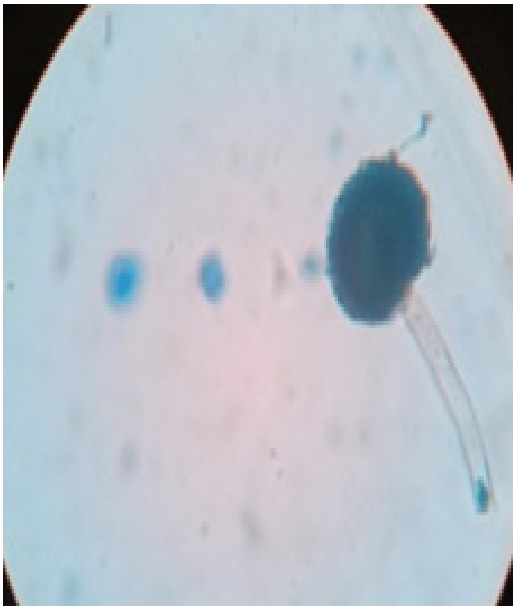


Fig 4 shows the growth of Asperigillus niger in Sabouraud dextrose agar culture and its microscopic appearance in Lactophenol cotton blue mount.

FIGURE - 5

ASPERGILLUS FLAVUS

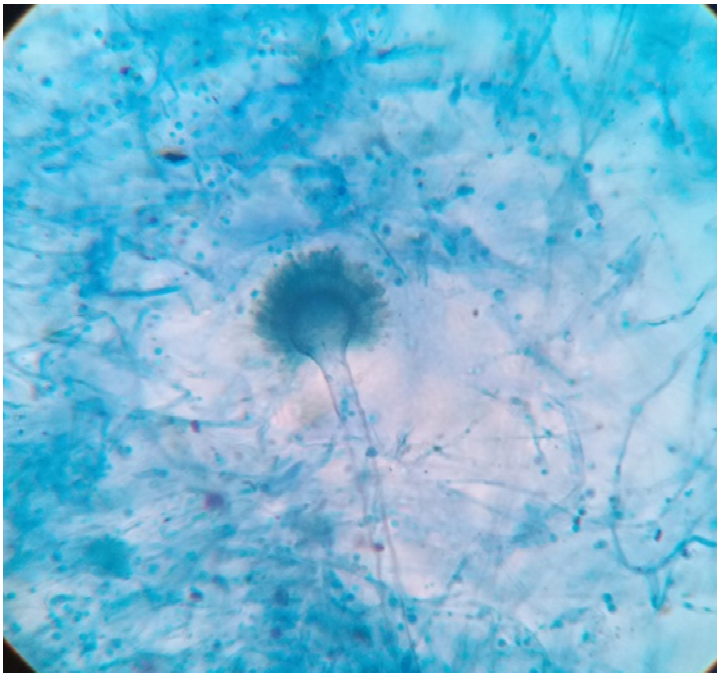


Fig 5 shows the growth of Asperigillus flavus in Sabouraud dextrose agar culture and its microscopic appearance in Lactophenol cotton blue mount.

FIGURE - 6

ASPERGILLUS FUMIGATUS

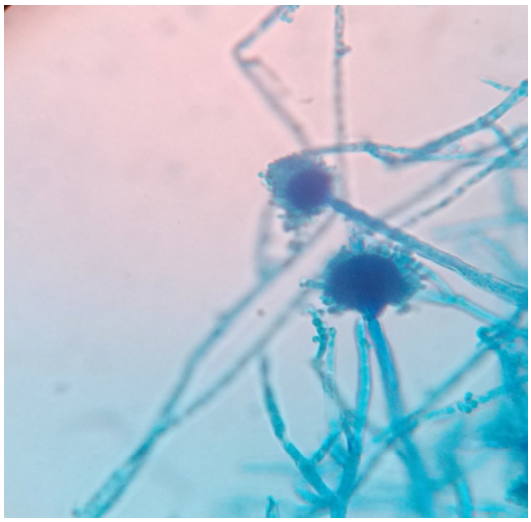


Fig 6 shows the growth of Asperigillus fumigatus in Sabouraud dextrose agar culture and its microscopic appearance in Lactophenol cotton blue mount.

FIGURE - 7

FUSARIUM

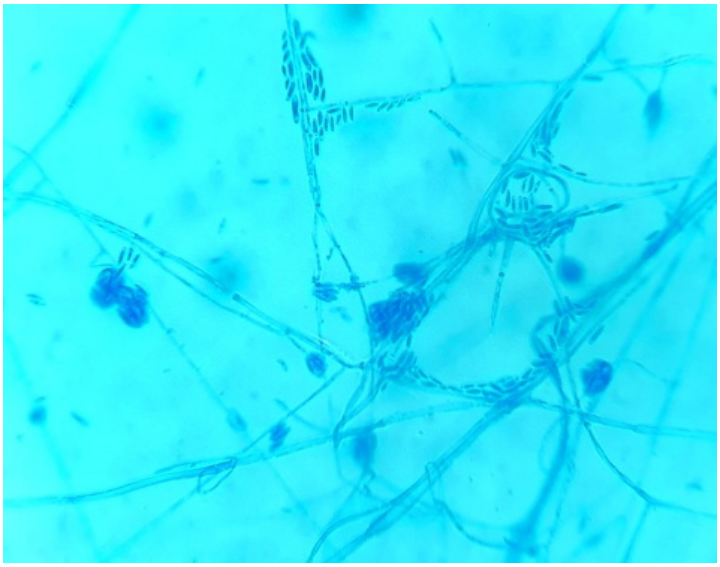


Fig 7 shows the growth of Fusarium in Sabouraud dextrose agar culture and its microscopic appearance in Lactophenol cotton blue mount.

FIGURE - 8

PENICILLIUM

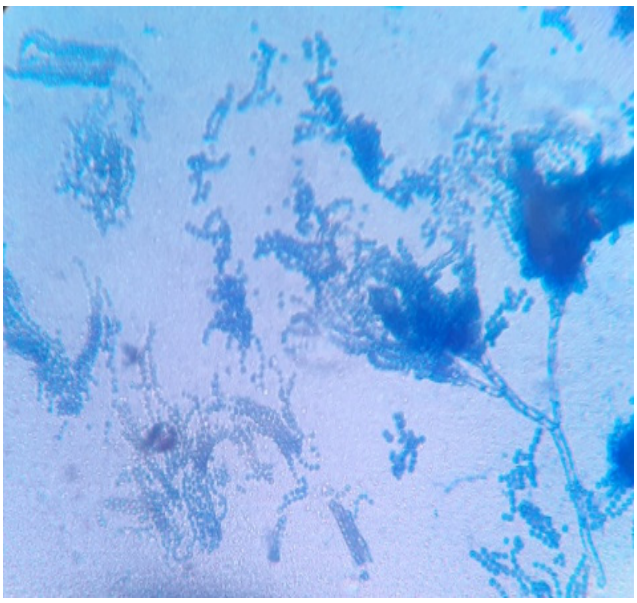


Fig 8 shows the growth of Penicillium in Sabouraud dextrose agar culture and its microscopic appearance in Lactophenol cotton blue mount.

FIGURE - 9

BROTH MICRODILUTION METHOD (MIC)

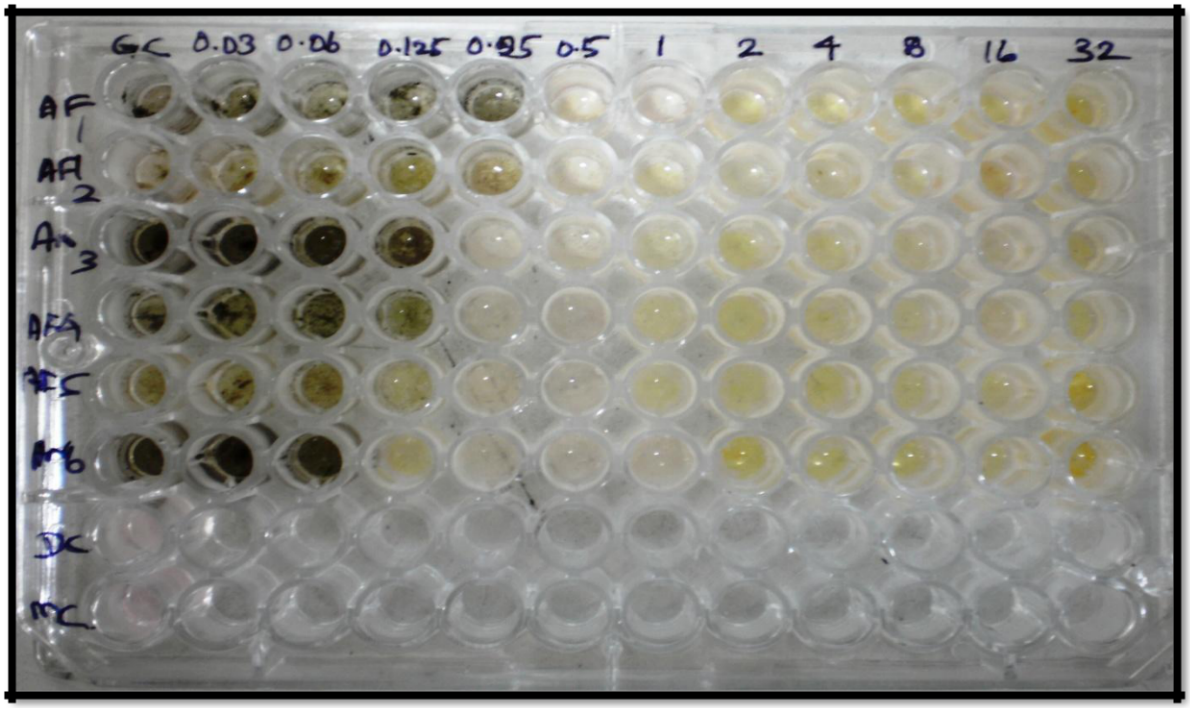


Fig 9 shows the detection of minimal inhibitory concentration (MIC) of antifungal drug to the fungal agent causing corneal ulcer by Broth microdilution method

DISCUSSION

DISCUSSION

A total of 100 patients with infectious corneal ulcer were selected for the study. 32 cases were culture positive (32%) (Table1). The cases were analyzed under the following parameters.

The age and sex distribution of infectious corneal ulcer was analyzed. 62 males and 38 females among these patients were studied. (Table 2). 94% (94/100) cases were found to be in age group between 10-60 years and 6% (6/100) of cases were in the age group of 51-60. Extremes of the age group showed low prevalence of corneal ulceration (Table3).

Considering the sex distribution (75%) males and (25%) female patients showed positive culture. A high prevalence of fungal corneal ulcers was seen among males contributing to 75 % of cases (Table2). Similar findings were observed in the study of Chowdhary et al 2005[17] which revealed higher prevalence (68%),among males. Lixen xie et al 2006[98] also reported male preponderance in the study. The age and sex distribution of the patients along with the positive culture for fungi were shown in Table 2&3. From this it seems that the maximum incidence of infected fungal corneal ulceration was in the 40 to 50 age group.

Considering the occupation, majority of the fungal corneal ulcer are seen in farm workers (53.12 %) (Table 9)

Among the fungal isolates out of 100 cases, 24 cases (75%) were due to Aspergillus species and next common agent isolated was Fusarium species 5 cases (15.63%) followed by Penicilllium species 2 cases (6.25%) and Curvularia species 1 case (3.13%).The distribution of fungal species were categorized in Table 4. The dominant role of Aspergillus species in corneal ulcer has been reported in the studies of Basak Samar K *et al* in 2005[9] and Khanal B *et al* in their study followed by Fusarium species[58]. In the study of Lixen *et al* in 2006 Prashant *et al* in 2007[98]. Fusarium species was found to be the most common fungi isolated. In this study Fusarium was isolated only in 15.63% of samples next to Aspergillus spp .This may be due to differences in climate and natural environment.

10% KOH mount preparation used as a screening test for rapid diagnosis of fungal corneal ulcer. Table 6 shows that out of the 33 samples showing the presence of fungal elements in KOH mount samples, 2 wear negative for culture. This correlates with the study of Vajpayee R B et al 1993[140] which revealed 94.3% sensitivity of 10% KOH mount examination Bharathi M J et al[13], 2007 reported 99% sensitivity and 1.5% false positive rate of KOH wet mount preparation.

Lactophenol cotton blue mount of 32 culture positive samples helped in microscopic identification of the fungal agents. Among the 32 culture positive samples, LPCB mount of 11 samples (34.38 %) showed Aspergillus fumigatus, 7 samples (21.88 %) showed Aspergillus flavus 6 samples (18.75 %) showed Aspergillus niger, 5 samples (15.63 %)showed Fusarium species, 2 samples (6.25 %) showed Penicillium species and 1 sample (3.13%) showed Curvularia species (Table 4). This is in accordance with the study in 2016 by Siva Prasad Basava et al, Efficacy of Lactophenol cotton blue for identification of fungal elements in Clinical Laboratory[118]

MIC determination by Broth microdilution method showed that 80.77% (21/24) of Aspergillus species, 80% (4/5) of Fusarium species, 100% (2/2) of Penicillium species and 100% (1/1) of Curvularia species showed sensitive range of MIC to Amphotericin B as showed Table 7. Totally 87.5% (28/32) of fungal isolates are sensitive to Amphotericin B.

For Itraconazole 70.83% (17/24) of Aspergillus species showed MIC less than 2µg/ml, 60 % (3/5) of Fusarium species, 100% (2/2) of Penicillium species and 100% (1/1) of Curvularia species showed sensitive range of MIC to Itraconazole (Table 8). Totally 71.88% (23/32) of fungal isolates are sensitive to Itraconazole. Ray A in 2002[104] studied the efficacy of Itraconazole showed 80% success rate of Itraconazole therapy in Aspergillus species.

SUMMARY

SUMMARY

Totally 100 infectious corneal ulcers were studied in detail. Aetiological fungal agents were isolated in 32/100 (32%) cases.

Among the fungal agents causing corneal ulcer, majority of the isolates were belonging to the genus Aspergillus (75%) followed by Fusarium(15.63%) , Penicillium species (6.25%) and Curvularia species (3.13%).

Male preponderance was observed (75%) in this study as compared to female (25%).

The age group most commonly affected was between 41 & 50 years which comprises 34.38% of total cases.

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 in the development of infectious fungal corneal ulcer.

Farm workers are the most commonly affected population by fungal corneal ulcer.

10% KOH mount is found to be highly sensitive as rapid screening tests for diagnosing fungal corneal ulcers.

Aspergillus species are the most commonly isolated agents from corneal ulcer patients (75%). In that Aspergillus fumigatus accounts for 34.38%, Aspergillus flavus accounts for 21.88% and Aspergillus niger accounts for 18.75%.

71.88% of fungal isolates were sensitive to Itraconazole and 87.5% of isolates were sensitive to Amphotericin B by Broth microdilution method.

CONCLUSION

CONCLUSION

The following are the conclusions derived from the present study on aetiopathogenesis of fungal corneal ulcers.

A variety of fungal isolates can cause infectious corneal ulceration in which Aspergillus fumigatus was the most common fungal species isolated which was susceptible to Amphotericin B and Itraconazole.

Among the various occupation, Farm workers are the major group of people affected by fungal corneal ulcers.

Among the sex distribution, Males are more commonly affected than female population which may be due to their occupational status and risk.

10% KOH mount seems to be the highly sensitive rapid screening test for diagnosing fungal corneal ulcers.

Culture (Sabouraud dextrose agar culture) followed by Microscopy (Lactophenol cotton blue mount) will provide confirmatory results in diagnosing the pathogens causing fungal corneal ulcer.

Most of the fungal isolates were sensitive to Amphotericin B and Itraconazole which can be used as an initial antifungal therapy after laboratory confirmation.

Precise identification of the causative organisms and timely institution of appropriate antifungal therapy based on the prevailing sensitivity pattern of fungal isolates will save the eye from microbial infection which is one of the preventable cause of blindness.

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ANNEXURES

ANNEXURE-1

STAIN & REAGENTS:

1. 10% KOH[49]:

Potassium hydroxide	:	10 g
Glycerol	:	10 ml
Distilled water	:	80 ml

2. GRAM STAIN[49] :

Methyl violet (2%)	:	10g methyl violet in 100 ml absolute alcohol 1 lit of distilled water (Primary stain)
Grams Iodine	:	10 g Iodine in 20 g KI (fixative)
Acetone	:	Decolorising agent
Carbol fuschin 1%	:	counter stain

3. LACTOPHENOL COTTON BLUE[50] :

For the staining and microscopic identification of fungi.

Cotton blue (aniline blue)	:	0.05 g
Phenol Crystals (C6H504)	:	20 g
Glycerol	:	40 ml
Lactic acid (CH3CHOHCOOH)	:	20 ml
Distilled water	:	20 ml

Method of preparation : This stain is prepared over two days

1. On the first day, dissolve the Cotton Blue in the distilled water. Leave overnight to eliminate insoluble dye.

2. On the second day, wearing gloves add the phenol crystals to the lactic acid in a glass beaker, place on magnetic stirrer until the phenol is dissolved.
3. Add the glycerol.
4. Filter the Cotton Blue and distilled water solution into the phenol/glycerol/lactic acid solution. Mix and store at room temperature.

MEDIA:

1.SABOURAUD'S DEXTROSE AGAR[50]:

Dextrose	**:**	20g
Neo Peptone	**:**	10g
Agar	**:**	20g
Distilled water	**:**	1000ml

pH : 6.8 ± 0.2

Suspend the ingredients in water, dissolve by heating to a boil and dispense in approximately 20 ml amounts in cotton plugged 25x150 mm test tubes with antimicrobial agent (Gentamicin 20 mg) added after heating the medium and before autoclaving at 121˚c for no longer than 15 minutes. Slant was allowed to harden and refrigerated. Cycloheximide was not added to the media since it is known to inhibit ocular fungal pathogen.

2. RPMI 1640 MEDIUM[50]: Commercially purchased RPMI 1640 media supplement with 0.3g of L-glutamate per litre without sodium bicarbonate (powder). Dissolve the powder in Nuclease free water. The medium was sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns. The pH was adjusted to 7.0. MOPS buffer was used.

PROFORMA

CASE HISTORY

Name :

Address :

Age :

Sex :

Occupation : Agriculturists/Athletes/Wrestlers/
Animal Keeping persons

Education :

Income :

IP No. :

Ward No. :

Diagnosis :

Date of admission :

Date of discharge :

Complaints :

Present history :

Past history :
Prior Treatment
Trauma
· Contact Lens wear
· Similar recurrent complaints
· H/O DM, HT, TB, Asthma.

GENERAL EXAMINATION :

SYSTEMIC EXAMINATION :

CVS :

RS :

Per abdomen :

CNS :

Ocular examination

Eye lid :

Conjunctiva :

Cornea :
Position, Size of Ulcer,
Margins,Slough,
Satellite Lesions,
Corneal sensation.

Anterior Segment
Examination :
Pupils :
Hypopyon :
Vision :

Investigation

· Syringing of nasolacrimal
Duct :

· Blood sugar :

TREATMENT DETAILS **:**Antibiotic/Topical steroidal eye drops immunosuppression / Any interventions / Duration of treatment

OUTCOME **: Cured / Improved/ Worsened /**

Death

MICROBIOLOGICAL REPORT

Specimen **: Corneal scraping**

Lab number **:**

Date **:**

Time **:**

TESTS DONE

A. KOH mount **:**

B. Culture

SDA **:**

C. LPCB MOUNT **:**

ANTIFUNGAL SUSCEPTIBILITY **:**

FINAL REPORT **:**

MASTERCHART

MASTER CHART

S.No	Name	Age	Sex	Occupation	IP / Op no	KOH	Culture	Sensitivity	
								Amphotericin	Itraconazole
1.	Ayyappan	45	M	Farmer	26568	-	-	-	-
2.	Muniyandi	52	M	Carpenter	21256	-	-	-	-
3.	Kandasamy	56	M	Coolie	26599	-	-	-	-
4.	Sengottayan	49	M	Contracter	22154	+	**Aspergillus fumigatus**	**S**	**R**
5.	Jagan	51	M	Farmer	23121	+	-	-	-
6.	Sundaram	45	M	Coolie	23455	-	-	-	-
7.	Kuppusamy	39	M	Farmer	22343	+	**Aspergillus flavus**	**S**	**S**
8.	Elumalai	55	M	Land owner	22367	-	-	-	
9.	Sadayappan	49	M	Farmer	23219	+	**Aspergillus niger**	**S**	**S**

10.	Sarasammal	45	F	Farmer	21133	-	**Aspergillus fumigatus**	**R**	**S**
11.	Navaneethan	41	M	Servant	21321	-	-	-	-
12.	Manikandan	59	M	Welder	22511	+	**Aspergillus flavus**	**S**	**S**
13.	Kanchana	36	F	Servant	23143	-	-	-	-
14.	Moorthy	43	M	Shopkeeper	23275	-	-	-	-
15.	Karuppanan	52	M	Shopkeeper	23341	+	-	-	-
16.	Chandrasekar	11	M	Student	23320	+	**Fusarium**	**S**	**S**
17.	Vasanthi	42	F	Teacher	21279	-	-	-	-
18.	Saravanan	22	M	Shopkeeper	24146	+	**Penicillium**	**S**	**S**
19.	Subramani	46	M	Coolie	25240	-	-	-	
20.	Marimuthu	56	M	Farmer	25333	+	**Aspergillus fumigatus**	**S**	**S**
21.	Jayamani	26	M	Farmer	25252	+	**Curvularia**	**S**	**S**
22.	Kumar	57	M	Driver	25239	-	-	-	-
23.	Selvam	48	M	Farmer	25277	+	**Aspergillus flavus**	**S**	**S**

24.	Karuppiyah	41	M	Saloon	25441	-	-	-	-
25.	Sekar	24	M	Workshop	26143	-	-	-	-
26.	Rani	35	F	Housewife	26151	-	-	-	-
27.	Anand	46	M	Farmer	25132	+	**Aspergillus fumigates**	**S**	**R**
28.	Nalini	41	F	Coolie	24131	+	**Aspergillus fumigatus**	**S**	**R**
29.	Munusamy	31	M	Farmer	24343	-	-	-	-
30.	Immanuvel	42	M	Farmer	23310	-	-	-	-
31.	Nandhini	49	F	Tailor	23750	-	-	-	-
32.	Dhandayutham	48	M	Landowner	24191	+	**Aspergillus flavus**	**S**	**S**
33.	Balan	11	M	Student	25936	-	-	-	-
34.	Kavipriya	43	F	Housewife	25834	-	-	-	-
35.	Ranganathan	41	M	Farmer	25781	-	-	-	-
36.	Karthikeyan	49	M	Driver	24323	-	-	-	-
37.	Kannammal	46	F	Housewife	24399	-	-	-	-
38.	Chinnasamy	39	M	Farmer	25311	-	-	-	-

39.	Venkatesan	33	M	Tailor	26131	-	-	-	-
40.	Ponnambalam	57	M	Carpenter	26332	+	**Aspergillus flavus**	**S**	**S**
41.	Veenarau	46	M	Welder	27341	-	-	-	-
42.	Somanathan	55	M	Farmer	27561	+	**Fusarium**	**S**	**R**
43.	Vetriselvi	47	F	Shopkeeper	27752	+	**Aspergillus niger**	**S**	**S**
44.	Suganthi	47	F	Housewife	28115	-	-	-	-
45.	Munusamy	38	M	Coolie	28834	+	**Aspergillus niger**	**S**	**S**
46.	Tamaraiselvan	55	M	Farmer	28264	-	-	-	-
47.	Parameshwari	57	F	Servant	28303	-	-	-	-
48.	Eswaran	49	M	Farmer	28354	-	-	-	-
49.	Muthu	54	M	Farmer	28423	+	**Aspergillus fumigatus**	**S**	**S**
50.	Ramu	53	M	Carpenter	28371	-	-	-	-
51.	Sekar	47	M	Coolie	28422	-	-	-	-

52.	Gayathri devi	24	F	Farmer	28437	+	**Aspergillus flavus**	**R**	**S**
53.	Baskaran	39	M	Carpenter	28754	-	-	-	-
54.	Jeevajothy	51	F	Coolie	28889	+	**Aspergillus fumigatus**	**S**	**S**
55.	Kalaiselvi	56	F	Housewife	28933	-	-	-	-
56.	Paneerselvam	48	M	Builder	28948	-	-	-	-
57.	Mayilvanan	42	M	Shopkeeper	28755	+	**Aspergillus niger**	**S**	**S**
58.	Thanigaivel	55	F	Housewife	28456	-	-	-	-
59.	Sumathy	38	F	Housewife	28936	+	**Aspergillus flavus**	**R**	**S**
60.	Diwakar	28	M	Farmer	28997	+	**Aspergillus fumigatus**	**S**	**R**
61.	Ganeshamoorti	47	M	Tailor	29313	-	-	-	-
62.	Kokila	25	F	Housewife	29416	-	-	-	-
63.	Mohanraj	45	M	Shop owner	29501	+	**Aspergillus niger**	**S**	**S**

64.	Poongothai	57	F	Housewife	29303	-	-	-	-
65.	Rathinam	40	M	Farmer	29305	-	-	-	-
66.	Ranjani	53	F	Housewife	29307	-	-	-	-
67.	Rani	54	F	Land owner	29401	-	-	-	-
68.	Anitha	36	F	Housewife	29409	-	-	-	-
69.	Leelavathy	54	F	Teacher	29511	-	-	-	-
70.	Selva	26	M	Student	29533	-	-	-	-
71.	Sasikala	51	F	Housewife	29545	-	-	-	-
72.	Lakshmi	58	F	Housewife	29571	-	-	-	-
73.	Selvi	55	F	Farmer	29580	+	**Fusarium**	**R**	**S**
74.	Christophy	38	M	Welder	29599	+	**Aspergillus fumigatus**	**S**	**R**
75.	Sripriya	48	F	Housewife	29655	-	-	-	-
76.	Vani	57	F	Housewife	29677	-	-	-	-
77.	Suseela	52	F	Coolie	29687	-	-	-	-
78.	Nandha kumar	49	M	Farmer	29699	+	**Fusarium**	**S**	**S**
79.	Kousalya	42	F	Farmer	29680	-	-	-	-

80.	Poovarasi	45	F	Anganwadi worker	29731	-	-	-	-
81.	Ponnan	30	M	Farmer	29800	-	-	-	-
82.	Mariyathal	48	F	Building worker	29888	-	-	-	-
83.	Mahalakshmi	52	F	Farmer	29999	-	-	-	-
84.	Ayyappan	36	M	Servant	30012	-	-	-	-
85.	Muniyandi	65	M	Farmer	30035	+	**Fusarium**	**S**	**R**
86.	Lakshmi	48	F	Housewife	30067	-	-	-	
87.	Kandasamy	56	M	Farmer	30088	+	**Aspergillus niger**	**S**	**S**
88.	Sengottayan	67	M	Land owner	30178	-	-	-	
89.	Jagan	55	M	Farmer	30188	-	-	-	-
90.	Sundaramal	42	F	Housewife	30197	-	-	-	-
91.	Kuppusamy	26	M	Servant	30277	-	-	-	-
92.	Elumalai	65	M	Farmer	30288	+	**Aspergillus Fumigatus**	**S**	**R**
93.	Balaji	62	M	Driver	30279	-	-	-	-

94.	Radhai	62	F	Housewife	30294	+	**Aspergillus Fumigatus**	**S**	**R**
95.	Sivakumari	47	F	Shopkeeper	30324	-	-	-	-
96.	Balu	32	M	Farmer	30356	+	**Penicillium**	**S**	**S**
97.	Neeraja	45	F	Teacher	30389	-	-	-	-
98.	Senthil	34	M	Farmer	30394	-	-	-	-
99.	Gayathri	54	F	Housewife	30412	-	-	-	-
100	Boomiraj	65	M	Farmer	30468	-	-	-	-

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ETHICS COMMITTEE CERTIFICATE

Name of the Candidate	:	Dr.K.R.Pandiaraj
Designation	:	PG in MD., Microbiology
Course of Study	:	2017- 2020
College	:	MADURAI MEDICAL COLLEGE
Research Topic	:	A study on isolation and characterization of fungal agents causing corneal ulcer in a tertiary care hospital
Ethical Committee as on	:	08.04.2019

The Ethics Committee, Madurai Medical College has decided to inform that your Research proposal is accepted.

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CERTIFICATE - II

This is to certify that this dissertation work titled "**A Study on isolation and characterisation of fungal agents causing Corneal ulcer in a tertiary care hospital"** of the candidate **Dr.K.R.Pandiaraj** with Registration Number **201714102** for the award of **M.D.,** in the branch of **MICROBIOLOGY**. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **4** percentage of plagiarism in the dissertation.

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