PATTERN OF FUNGAL CORNEAL ULCERATION AT TILGANGA INSTITUTE OF OPHTHALMOLOGY

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ABSTRACT

Corneal ulcer is a leading cause of ocular morbidity and blindness worldwide. Among the corneal diseases, fungus keratitis or fungal corneal ulcers form major blinding eye disease. This study was conducted to isolate and identify fungal pathogens involved in corneal ulcers. A total of three hundred corneal scrapings were collected from the suspected patients visiting Out Patients Department ward of Tilganga Institute of Ophthalmology from July 2010 to February 2011 and subjected to microscopic observation and culture. Corneal scrapings were smeared onto three slides to prepare 10% KOH wet mount or Lactophenol Cotton Blue stain, Gram's stain and Giemsa stain. Also, samples were streaked directly onto the surface of Sabouraud's Dextrose Agar, Blood Agar, Chocolate Agar and in Thioglycolate Broth which were incubated at 27°C in aerobic condition for 7 days to 28 days and these plates were observed for fungal growth.

Out of 300 samples collected, 21% (n=63) samples had shown growth of fungal pathogens. Out of 63 fungal isolates, majority of the isolates i.e. 38.1% (n=24) were identified as *Aspergillus* spp followed by *Fusarium* spp 19% (n=12) and *Curvularia* 14.3% (n=9) respectively. Other fungal isolates included *Cladosporium* (7.9%), *Penicillium* (7.9%), *Acremonium* (4.8%), *Bipolaris* (3.2%) *Candida albicans* (3.2%) and *Rhizopus* (1.6%). Altogether, 52.7% (n=158) samples were collected from male patients and 47.3% (n=142) samples were collected from female patients. Rate of infection was found to be higher in males (27.2%) than females (14.1%) but there was no significant difference between the males and females (p=0.157). The mean age calculated was 44.8 years in male and 41.7 years in female. The highest number of fungi was isolated from 31-40 (23.8%) age group followed by 51-60 (22.2%) age groups. Agricultural worker group were mostly infected. The rate of infection was higher in the months of November and December.

Filamentous forms of fungi were found in higher number than non- filamentous forms. Among the filamentous fungi, *Aspergillus* was the most frequently isolated followed by *Fusarium* and *Curvularia* respectively. *Aspergillus fumigatus* (62.5%) was the most frequently isolated *Aspergillus* species.

Key words: corneal ulcer, corneal scraping, filamentous fungi, *Aspergillus*, *Fusarium*

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LIST OF ABBREVIATIONS

ATP	:	Adenosine Tri Phosphate
BA	:	Blood Agar
CA	:	Chocolate Agar
CO_2	:	Carbon Dioxide
D/W	:	Distilled Water
H & E stain	:	Haematoxyline and Eosine stain
KOH	:	Potassium Hydroxide
LPCB	:	Lacto Phenol Cotton Blue
MMP	:	Matrix Metalloproteinases
NA	:	Nutrient Agar
OPD	:	Out Patients Department
PDA	:	Potato Dextrose Agar
SDA	:	Sabouraud Dextrose Agar
SPSS	:	Statistical Package for Social Services
TIO	:	Tilganga Institute of Ophthalmology
UK	:	United Kingdom
USA	:	United States of America
Vs	:	Verses
WHO	:	World Health Organization

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

The inflammation of the cornea is known as keratitis. The keratitis can be either ulcerative or non ulcerative. Clinically, ulcerative keratitis is graded as mild, moderate or severe depending on the size of the ulcer, depth of the ulcer, infiltrate depth and density and involvement of sclera. A corneal ulcer is an inflammatory condition of the cornea involving distribution of its epithelial layer with involvement of the corneal stroma and mostly accompanied by photophobia, lacrimation, pain and dimunation of vision. According to the location of the ulcer, it may be central, paracentral, and central peripheral or total (Prajna *et al*., 2008).

The World Health Organization has recognized the diseases of the cornea as one of the major causes of vision loss and blindness, next to cataract blindness. It is estimated that ocular trauma and corneal ulceration result in 1.5 to 2 million new cases of corneal blindness every year (Pranja *et al*., 2008). Corneal ulcer is one of the major public health problems in developing countries like Nepal. Suppurative corneal ulcer is still one of the leading causes of blindness with an annual incidence of 799 cases per 1,00,000 populations in Nepal, of which nearly 22% are of fungal origin (Upadhyay *et al.,* 2001).

Corneal ulcer generally results from bacterial, protozoal, viral or fungal infection. Among the corneal diseases, fungus keratitis or fungal corneal ulcers form major blinding eye disease in Asia (Lalitha *et al*., 2008). As fungi are a part of normal microbial environment, they rarely cause infection in human cornea in the absence of a precipitating event. More than 105 species of fungi classified in 56 genera have been reported to cause keratomycosis. *Aspergillus* spp, *Fusarium* spp, *Curvularia* spp, *Penicillum* spp, *Rhizopus* spp, *Candida* spp, *Alternaria* spp, *Cephalosporium* spp, *Cladosporium* spp, *Mycosis fungoides*, *Paecilomyces* spp, *Phialophora* spp, *Nocardia*, *Actinomyces*, *Mucor* etc are some of the fungi which cause corneal ulcers (Prajna and Venkatesh, 2002). Among such fungi filamentous forms are the major etiologic agents involved in corneal ulcers.

Multiple predisposing factors are responsible for the causation of fungal corneal ulcers. Use of corticosteroids or antibiotics, extraocular foreign bodies, trauma, post operative infections, pre existing eye diseases, contact lens, systemic illness, age, sex,

climate and seasons, abrasion, burns and chemical injuries, corneal surface disorders, dry eye, exposure keratitis etc are the predisposing factors to cause fungal corneal ulcers (Sirikul *et al*., 2008). The contamination of donor corneas with fungal organisms may also cause fungal corneal ulcers. Trachoma, neonatal ophthalmia, xerophthalmia, and onchocerciasis (in Africa), harmful eye practices by patients, village healers, and poorly trained medical personnel were considered as responsible factor for high prevalence of corneal scars in developing countries in the past (Whitcher and Srinivasan, 1997). However, recent evidence suggests that primary corneal ulceration is also very important causes of corneal scarring and visual loss in developing countries. Fungal keratitis is particularly common in agricultural societies where trauma with vegetable matter or organic matter often leads to corneal ulceration. The traumatic agents include paddy, maize, grass, wooden, dust, injury with tail of domestic animals like cow, buffalo, beak of hen, finger nail etc. Fungal keratitis is common in the season of harvesting crops because of a larger number of vegetative injuries and early winter seasons and monsoon season because of high humidity, coldness, dryness and windy climate found in these months. Filamentous fungal keratitis can occur after surgery such as penetrating keratoplasty and radial keratoplasty (Polack *et al*., 1991).

The clinical appearance of fungal corneal ulcer varies greatly depending on the type of organisms, duration of the disease and the severity of infection. It may occur as quickly as 24-48 hrs or may be delayed for 10-20 days. The characteristic clinical manifestation include dry raised surface, infiltrate with feathery margins, satellite lesions, posterior corneal abscess and a thick endothelial exudates. Symptoms of corneal ulceration include the presence of pain, watering, redness, discharge, photophobia, decrease in visual acuity and swelling of lids (Prajna *et al*., 2008).

Prompt and appropriate anti-fungal therapy is the mainstay of the treatment of fungal corneal ulcers. Since the corneal epithelium serves as a barrier to the penetration of most topical antifungal agents, debridement of the corneal epithelium is an essential component of the medical management. Some of the topical agents used are natamycin 5% suspension, amphotericin B, fluconazole, econazole, clotrimazole, voriconazole etc. The duration of treatment is longer than that of bacterial keratitis (Pachigolla *et al*., 2007). In addition to antifungal drugs a broad spectrum antibiotic should be given to prevent secondary bacterial infection. Fungal corneal ulcers occur in healthy immunocompetent individuals. However, once the corneal ulcer perforate

further complications are added, vision is lost and there is irreversible damage to the eye. So, people are advised to wear protective glass wares during their work at agricultural fields. The importance of health education to the general population mainly to farmers should be emphasized. A similar study was done by Ganguly *et al*., 2011 and Feilmeier *et al*, 2010 in which *Fusarium* and *Aspergillus* were found to be the common fungal isolates causing corneal ulcer in Nepal respectively.

Nepal is an agricultural country; many Nepalese people are the victims of fungal corneal ulcers due to trauma with agricultural products and other risk factors. Very limited study has been done in fungal corneal ulcers in Nepal. The purpose of this study is to assess the prevalence of specific fungal pathogens and to analyze the influence of demographic factors on the spectrum of fungal species responsible for fungal corneal ulcers. So this study is mainly focused on bringing awareness towards ocular fungal infections as it may cause the loss of vision permanently if not treated on time.

1.2 Objectives

1.2.1 General objective

To isolate and identify the fungal pathogens involved in corneal ulcer in patients visiting Tilganga Institute of Ophthalmology, Gaushala, Kathmandu

1.2.2 Specific objectives

i) To describe the distribution pattern of fungal pathogens in corneal ulcers

ii) To isolate and identify the predominant fungal genera amongst the different isolates

iii) To describe month wise and occupation wise distribution of fungal corneal ulcer cases

CHAPTER II
LITERATURE REVIEW

2.1 Corneal anatomy of human

Cornea is a transparent avascular tissue with smooth surface and richly supplied by ciliary nerves. It is a clear 'window' in front of the iris, pupil and anterior chamber of the eye. The cornea is slightly oval. It is thicker at the periphery than at the centre. Transparency of cornea is due to the uniform and perfectly organised distribution of the collagen fibrils within the stroma and this property allows light to reach the retina for the functioning of the eye (Khurana and Khurana, 2008). Histologically, cornea has five distinct layers from anterior to posterior.

Epithelium: It is a continuation of the epithelium of the bulbar conjunctiva and consists of five layers of cells. It constitutes 10% of the total wet weight of the cornea. It has smooth refractive surface and protects against infection. The epithelium contains ATP, glycogen and ascorbic acid. Acetylcholine and cholinesterases are also present in high level in epithelium. Perhaps these play a role in cation transport as well as in trophic nerve function.

Bowman's membrane: It is a thin structureless layer of about 12µm thickness, placed between the epithelium and corneal stroma. It is not a true elastic lamina. Once destroyed it does not regenerate. However, it shows a considerable resistance to infection. It is rich in fibronectin and formed from secretion from both basal epithelial cells and keratocytes.

Corneal stroma: It constitutes 90% of the entire thickness of the cornea and is composed of a modified connective tissue containing lamellae and cells. Corneal stroma constitutes collagen fibrils embedded in hydrated matrix of proteoglycans. Collagen constitutes approximately 70% of dry weight of human cornea. The corneal collagen is dissolved by proteolytic enzymes such as collagenase, which has important implication in corneal ulceration. It has matrix metalloproteinases (MMP) enzymes which break down components of the extracellular matrix and help to maintain the normal corneal structure. Hence it plays a critical role in restructuring the cornea after injury.

Descemet's membrane: It is made up of collagen about 73% and glycoproteins. The collagen of descemet's membrane is insoluble and extremely resistant to chemical and enzymatic action than the corneal stromal collagen, these accounts for the resistance offered by descemet's membrane to trauma, chemical agents, infection and a barrier to perforation in deep corneal ulcers.

Endothelium: It is made up of monolayer of interdigitating hexagonal cells joined by tight junctions. It transports nutrients into cornea and also pumps fluid out of cornea. It acts as barrier between stroma and anterior chamber; keeps cornea dehydrated and clear. The histochemical examination of the endothelium has shown the presence of enzymes needed for Glycolysis and Kreb's cycle (Gupta *et al*., 1998).

2.2 Defense mechanism of the eyes

Anatomically, the eye is protected from the introduction of microbes due to trauma by the surrounding bony structure of the protruding orbital rim. The cilia protect the eyelid by a rapid blink reflex. The lashes entangle the microorganisms and prevent them from entering the conjunctival sac. The eyelid skin, cilia and adnexal surfaces are normally inhabited by non-pathogenic, saprophytic aerobic and anaerobic bacteria or fungi which decrease the chances of colonization by the pathogenic microbes. Cornea, conjunctiva and tear film act as major component of ocular defense system against the microbial infections. There exists a good balance between the cornea and its environment that helps the cornea to maintain its integrity in spite of continuous exposure to foreign bodies and pathogens. The normal cornea is protected from the injurious agents in the event by blinking reflex of the eyelids, constantly reformed pre corneal tear film, the intact epithelium and bowman's membrane. The cornea is equipped with a protective lining composed of two layers of mucosubstances, the

glycocalyx layer and the mucus layer produced by globet cells of the conjunctiva. The corneal epithelium acts as a mechanical barrier, the cellular and chemical components of conjunctiva and pre-corneal tear film acts as biological protective system. Any imbalance in the interplay of these factors due to malnutrition, systemic disease, injury or local change in the micro environment can result in the corneal ulceration (Panda, 2007). There are multiple barriers to ocular infections. The presence of an intact tear film and its drainage by the lacrimal apparatus acts as an intrinsic barrier to infection. The microorganisms, foreign bodies and desquamated epithelial cells are continuously washed out of the eye due to blinking and lacrimal drainage system. The mucus layer of the tear film also provides antimicrobial properties which inhibits the bacterial and fungal adhesion to the epithelial cell layer. Mechanical action of lid movement keeps the conjunctiva moist and well lubricated. Other molecules which provide antimicrobial protection are microbe-specific antibody (IgA) and non-specific antimicrobial molecules such as complement, lactoferin, lysozyme and β-lysin. Cell-dependent cellular immunity defends the host cells. Neutrophils and monocytes also damage the fungus by their myeloperoxidase and hydrogen peroxide. Monocytes damage fungi by oxidative mechanism. Failure of any of the above defense mechanisms predisposes ocular infections (Mukharjee and Bandyopadya, 2010).

2.3 Microbiology in relation to the eyes

The eyes are sterile only for few hours after the birth and then get invaded by microorganisms. The eyes may be infected during the birth if the labor is prolonged. The eyes being exposed to the atmosphere throughout the day and their continuity with skin, nasopharynx makes them vulnerable to contamination and keep them contaminated. The organisms are found on the skin of the lids, the lid margins and conjunctiva in abundance. The cornea rarely yields microorganisms unless diseased. The organisms that are known to cause ocular infections are bacteria, chlamydiae, fungi, virus, parasites, arthropods, and larvae etc. These organisms can be either saprophytes or commensals. They have tendency to cause opportunistic infection whenever the conditions are in favour of the organisms and against the host. Microfloras are generally similar in both eyes at onset, course and type. The routes of ocular infection can be exogeneous, endogeneous or through inoculation. The exogeneous routes include air, water, body fluid, infected drops, instruments, contact lens, foreign bodies and fomites. The endogeneous route is through blood borne.

Penetrating injuries, surgical wounds, retained foreign body, viscoelastic irrigating fluids, perforating corneal ulcers are the inoculation routes of ocular infection. Rich blood supply of the eye except the cornea and lens makes the eye predisposed to haematogenous spread of the infection. The meninges and cerebrospinal fluid (CSF) transport intracranial infection to the eye. The organism finds the thin walls of paranasal sinuses easy to pass through (Mukherjee and Bandyopadya, 2010).

Some of the microfloras found in the eyes are as follows:

Staphylococcus aureus and other species of Staphylococci

Haemophilus influenzae
Streptococcus pneumoniae
Bacillus species
Corynebacterium species
Pseudomonas species
Neisseria species
Aspergillus species
Fusarium species
Rhizopus species
Curvularia species etc.

Organisms isolated from cornea may not be the same as those from conjunctiva or lacrimal sac (Panda, 2007).

2.4 Diseases of the cornea

Diseases of the cornea are extremely serious because they disturb the transparency that leads to permanent visual impairment ranging from slight blurring to total blindness. The diseases of the cornea may be inflammatory, degenerative, symptomatic or developmental in origin (Nema and Nema, 1998).

a. Corneal ulcer: It is an inflammatory condition of the cornea involving disruption of its epithelial layer with involvement of the corneal stroma. It is common condition in human particularly in the tropics and the agrarian societies. Many microorganisms cause infective corneal ulcer. Among them are bacteria, fungi, viruses, protozoa and chlamydia. Bacterial keratitis is caused by *Staphylococcus aureus, Streptococcus viridans, Escherichia coli, Enterococci, Pseudomonas, Nocardia* and many other bacteria. Fungal keratitis causes deep and severe corneal ulcer. It is caused by *Aspergillus* spp, *Fusarium* spp, *Candida* spp, *Rhizopus, Mucor* and other fungi. The typical feature of fungal keratiits is slow onset and gradual progression, where signs are much more than the symptoms. Viral keratitis is caused by Herpes simplex, Herpes Zoster, Adenoviruses, Coronaviruses and many other viruses. Herpes virus cause a dendritic ulcer, which can recur and relapse over the lifetime of an individual.

Protozoa infection like *Acanthamoeba* keratitis is characterized by severe pain and is associated with contact lens users (Hamana and Kaufman, 1987). *Chlamydia trachomatis* can also contribute to development of corneal ulcer.

b. Hypopyon ulcer: A disc shaped central corneal ulcer with hypopyon (sterile pus in the anterior chamber) and violent iridocyclitis is called hypopyon ulcer. It is usually found in old, debilated, malnourished patients who may be having dacryocystitis. There is always a risk of development of hypopyon ulcer following an injury by organic matters like leaf, twigs, coal, stone and finger nail. *Pneumococcus, Streptococcus, Gonococcus* and *Proteus vulgaris* are common pyogenic organisms capable of producing the ulcer. *Pseudomonas pyocyanea* causes a fulminant sloughing corneal ulcer with a greenish look within a short time. Hypopyon is frequently found in mycotic corneal ulcers.

c. Marginal corneal ulcer: It is frequently seen in old people and is caused by *Staphylococcus, Morax-Axenfeld* diplobacilli, *Haemophilus aegyptius*. It is often associated with chronic blepharo-conjunctivitis. It is caused by hypersensitivity reaction to the exotoxins of *Staphylococcus*. It appears as shallow infiltrated crescents at the corneal periphery with vascularisation and runs a chronic indolent course but seldom perforates.

d. Mooren's ulcer: It is rare disorder of unknown aetiology. Recently it is considered as a peripheral corneal melting syndrome probably due to an ischaemic necrosis resulting from limba vasculitis. Collagenase and proteoglycanase are produced by the conjunctiva adjacent to the ulcer. It is usually unilateral, superficial and often affects elderly people.

e. Atheromatous corneal ulcer: It generally occurs in an old leucoma undergoing degenerative changes with calcareous deposits. As the cornea is devitalised and insensitive, it is readily vulnerable to infection. The ulcer perforates quickly and panophthalmitis supervenes.

f. Corneal ulcer due to malnutrition: Malnutrition lowers the general body resistance and predisposes the individual to infection. Central marasmic ulcer and keratomalacia are two types of corneal ulcers found in poorly nourished children. Bilateral, central, superficial or deep corneal ulcer with vascularisation may occur in marasmic children and is prone to rapid perforation. Keratomalacia is characterized by bilateral melting of the cornea associated with xerosis of the conjunctiva and avitaminosis A. It results from vitamin A deficiency.

An ulcer of the cornea heals by the migration of surrounding epithelial cells followed by mitosis of the cells and by the introduction of blood vessels from the conjunctiva. Superficial small ulcers heal rapidly by the first method. However, larger or deeper ulcers often require the presence of blood vessels to supply inflammatory cells. White blood cells and fibroblasts produce granulation tissue and then scar tissue, effectively healing the cornea.

2.5 Predisposing factors

Various risk factors for the causation of fungal corneal ulcer may be ocular or related to systemic status of the patient.

2.5.1 Ocular factors

Ocular factors that may predispose to the occurrence of fungal corneal ulcer include trauma, contact lens wear, medications and prior ocular surgery.

Trauma: It often occurs outdoors and involves plant matter such as leaves and paddy grains (Chander and Sharma, 1994). It may also occur after injury with mud or sand and even injury from animal origin such as due to cow dung, cow tail and may occur even with metal pieces. Fungal keratitis should be especially suspected in children in cases when trauma with organic matter occurs (Upadhaya *et al*., 2001). Traumatic corneal ulcer is an important cause of bilateral and monocular blindness in the developing world, with estimates of 5% of all blindness being trauma related (Hall and Lion, 2005). The retained intraocular and intraorbital foreign bodies are constant and common sources of ocular infection.

Contact lens wear: Fungal corneal ulcer following contact lens wear is rare. Filamentous fungi are more commonly associated with cosmetic lens wear, and yeasts are more frequently associated with therapeutic lens use (Sweeney *et al*., 2003). The organisms can adhere to contact lens surfaces or may be present in non- sterile fluid used to rinse contact lens.

Drugs: The indiscriminate use of topical antibiotics and topical corticosteroids has been associated with fungal keratitis especially in developing countries where the drugs are available over the counter without any prescription (Varenkar *et al.,* 1991). They appear to activate and increase the virulence of fungi. The infiltrates are more extensive in such cases and response to anti-fungal therapy is more sluggish. Systemic use of corticosteroids may predispose the patient to fungal keratitis as the immune

response of the host is suppressed. The use of traditional eye medicines may also predispose to the occurrence of fungal keratitis.

Corneal surgery: Fungal corneal ulcer may occur virtually after any ocular surgery which includes keratoplasty, cataract surgery, and refractive surgery. Predisposing factors for the development of fungal keratitis in patients after penetrating keratoplasty include suture related problems, topical steroids and antibiotic use, failed grafts and persistent epithelial defects. The contamination of donor corneas with fungal organisms may occur as no anti-fungal therapy is routinely used to decontaminate the donor eyes (Wang *et al.,* 2000).

2.5.2 Systemic factors

Some systemic diseases associated with immunosuppression may increase the risk for the development of fungal keratitis. These include diseases such as diabetes mellitus, patients with chronically debilated diseases who are hospitalized in the intensive care units, HIV positive patients (Hemedy, 1995) and cases of leprosy. Vitamin A deficiency is another very important and common cause of childhood blindness in the developing countries (Sherpa *et al*., 2011). Conjunctivitis is found to be the most frequent diseases predisposing to corneal ulceration. Poor general hygiene is also found to be the most frequent systemic predisposing factor.

2.5.3 Others factors

Age: Fungal corneal ulcer is more common in the working age groups (20 years to 60 years), it has been reported in extremes of age also.

Sex: Fungal corneal ulcer does not have any gender predilection, although it is found more in male patients due to their maximum involvement in outdoor activities compared to the female ones who are busy with their indoor activities.

Occupation: Farmers and the people who work in agricultural fields are more prone to the infection.

Seasonal variation: Higher cases of fungal keratitis are occurred during monsoons and early winter because of the high humidity found during these months. A higher incidence has also been reported during harvest seasons, springs, and early winter, probably because of a larger number of vegetative injuries during these seasons (Sharma and Vajpayee, 2008).

2.6 Pathogenesis of corneal ulcers

Fungal infections involve adaptive interactions between host and pathogen. To infect tissues, fungi adjust to local conditions such as ambient pH, temperature and nutrient supply. Fungi that are pathogenic for the eye have the capabilities of colonizing and invading the injured ocular surface through mechanisms that enable growth and survival (Rohini *et al*., 2007). Fungi produce trichothecenes toxin. They also produce proteolytic enzymes and an inhibitory factor which inhibits neutrophils. The inflammatory reaction results from replicating and non-replicating fungi, mycotoxins, proteolytic enzymes and soluble fungal antigens. Corneal ulcer passes through the different stages. In the first stage, which is known as progressive stage, adherence and entry of fungi are followed by invasion into the stroma. They release toxin and enzymes and cause tissue destruction. This leads to necrosis and sloughing of the epithelium, bowman's membrane and the involved stroma. In active ulceration stage, pain, photophobia, decreased visual acuity, necrosis and subsequent sloughing of the epithelium and stroma, infiltration of polymorphonuclear leucocytes, edema of the adjacent epithelium and stroma occurs. The necrotic base of the ulcer is surrounded by heaped-up tissue. Anterior chamber inflammation may progress to a large hypopyon. The ulcer may penetrate deeper into the cornea and lead to formation of a descemetocele. The ulcer may also progress peripherally towards the sclera. In the regressive stage, humoral and cellular immune defenses combine with antimicrobial therapy to limit proliferation of the fungi, kill fungi, and allow phagocytosis of the fungi and cellular debris. A distinct line of demarcation may develop as the ulcer and stromal infiltration round up their edges. Vascularization of the cornea may also occur. In the healing stage, the epithelialization process starts. The histiocytes and keratocytes convert to fibroblasts so that the scar tissue is formed. Vascularization occurs towards the ulcer sites, which further promotes healing as a result of influx of fibroblast and antibodies. When the healing is complete, the vessels regress and become 'ghost vessels' which may be visualized by indirect illumination. White scarring varies in amount and becomes translucent with time (Sharma and Vajpayee, 2008).

Histopathologic changes in mycotic keratitis were first described by Neumann and associates. They highlighted certain characteristic features such as the presence of fungi throughout the corneal thickness and their absence from the corneal surface or ulcer crater in most cases (Hall and Lion, 2005). However, all such incidents do not result in ulcer unless there is a breach in the corneal epithelium; delay in healing that

provides the ‘window of opportunity’, lodgment of the microorganisms and development of subsequent ulceration. Often it may be difficult to ascertain whether the sequel of corneal ulceration in this group of patients is because of direct trauma or because of the poorly healing epithelium (Chatterjee and Agrawal, 2010). The clinical diagnosis of microbial keratitis often relies on a history of infectious exposure and the morphological features of corneal inflammation. Ophthalmologists use clinical clues to recognize ocular surface infection, and some distinctive though not pathognomic signs may help to differentiate bacterial, fungal and amoebic pathogens of cornea (Dahlgren *et al*., 2007). The classical symptoms of corneal ulceration include the presence of pain, watering, discharge, photophobia, decrease in visual acuity and swelling of lids. In general, superficial ulcers are more painful than deep corneal ulcers. The type of causative organisms and the depth of the ulceration influence the severity of pain. An associated conjunctivitis may cause severe redness as in gonococcal, pneumococcal and *Haemophilus* infections. Almost all cases of corneal ulcer present with a complaint of discharge from the affected eye. The extent of decrease in vision depends on duration, severity and location of the lesion, involvement of ocular structures. In a case of active keratitis the precorneal tear film and meniscus typically consist of numerous cells and debris unlike an inactive corneal ulcer. A beach in the corneal epithelium is more characteristically seen in cases of active keratitis unlike a non- infective infiltrate where the overlying epithelium is intact. However, in some cases of deep ulceration, the overlying epithelium may be completely intact. The examination of the cornea includes the examination of the corneal ulcer as well as the examination of the surrounding cornea. On examination under the slit-lamp location, shape, margins, size, epithelial defect and infiltration of the corneal ulcer should be recorded (Sharma and Vajpayee, 2008).

2.7 Fungi

Even though there exist over 100,000 species of fungi, only a few of these are known to be pathogenic for human (Pelczar *et al*., 2004). Fungi are eukaryotic chemorganotrophic organisms that possess no chlorophyll and are abundant in soil and air. They live as saprophytes or parasites over dead or living organisms. Saprophytes decompose complex plant and animal remains, breaking them down into simpler chemical substances that returned to the soil, thereby increasing its fertility. Thus they can be quite beneficial to humans and are also important in industrial

fermentation. Fungi cause diseases in plants, humans and other animals as parasites. Although fungal diseases are less commonly encountered than bacterial or virus diseases in humans and other animals, they are of great importance in causing diseases of plants. Their importance as a cause of disease in plants and animals was established earlier than bacteria or viruses. The fungi differ from bacteria in that they possess rigid cell wall containing chitin, mannan or other polysaccharides, true nuclei, nuclear membrane, paired chromosomes, mitochondria, ribosomes and food reserve. They divide asexually, sexually or by both processes. They may be unicellular or multicellular. Pleormorphism is common. They have the ability to grow at body temperature, survive at the low redox potential of tissues and neutralize the humoral and cellular defenses of the host. The fungi belong to phylum Thallophyta. The fungi are divided into pseudomycetes and eumycetes. The pseudomycetes are now considered to be higher bacteria. The eumycetes can be with septate hyphae or without septate hyphae (Brooks *et al.,* 2004; Greenwood *et al*., 2006).

According to the morphology of the fungi, they are divided into four types:

1. Moulds filamentous fungi: They form true mycelium and reproduce by the formation of different types of spores such as arthospores, chlamydospores and conidia. For example dermatophytes.

2. Yeasts: They are unicellular, occur as spherical or ellipsoidal cells and reproduce by simple division, budding. For example genus *Cryptococcus.*

3. Yeasts like fungi: They grow partly as yeast and partly as elongated cells with lateral budding resembling hypha to form pseudomycelium. For example *Candida albicans*.

4. Dimorphic fungi: They can exist as filaments or yeasts at different temperature. They are moulds in soil and in culture but in host body they appear like yeast. Most fungi causing systemic infections are dimorphic.

The spores are the reproductive parts in fungi. On the basis of spore formation, the fungi are divided into four classes:

1. Phycomycetes: They are lower fungi, have non-septated hyphae. They have both types of reproduction, i.e. asexual and sexual. They have endogeneous spores. The asexual spores are called sporangiospores. The sexual spores are either oospores or zygospores.

2. Ascomycetes: They have exogeneous sexual spores called ascospores, they reproduce asexually.
3. Basidiospores: They form sexual spores called basidiospores.
4. Deuteromycetes: They are also known as fungi imperfecti. The sexual phase of these organisms has not been known (Smith and Charles, 1990).

2.7.1 Fungi of ocular importance

Fungi are normal inhabitants of conjunctival sac (2-20%). They are more common in inflammation (40% or more) and further increase after local use of antibiotics or corticosteroids. Fungi can be isolated from the flora of the normal eyelid and conjunctiva in individuals who work outdoors. Keratomycosis is common in tropical climatic regions and is a common cause of corneal ulcer. More than 105 species of fungi classified in 56 genera have been reported to cause oculomycosis. Many fungi involved in ocular infections are saprophytic in nature. Opportunistic fungi rarely affect intact cornea, however they become pathogenic in a compromised or immunocompromised state. Besides cornea, fungi can also affect skin of lids, conjunctiva, iris, vitreous, lacrimal sac, and orbit.

Fungal corneal ulcer or fungal keratitis was first described by Leber in 1879 (Thomas, 2003a). Keratomycotic fungi can be broadly classified into filamentous fungi, yeasts, or dimorphic fungi (Sharma and Vajpayee, 2008).

2.7.1.1 Filamentous

Filamentous form of keratomycotic fungi can be classified into septated and non-septated fungi.

A. Septated

Septated fungi can again be classified into non- pigmented and pigmented septated fungi.

1. Non-pigmented

Fusarium solani, F. oxysporum, F. moniliforme

Aspergillus fumigates, A. flavus

Acremonium (cephalosporium)

Paecilomyces

Penicillium

2. Pigmented (Dematiacious)

Curvularia senegelensis, C. verrulosa

Lasiodiplodia

Alternaria

Cladosporium

Celletotrichum

Drechslera (Helminthosporium)

B. Non- septated

Rhizopus (mucormycosis)

A. Septated

1. Non-pigmented fungi

Some of the important characteristics of non-pigmented, septated filamentous fungi are described below:

a. ***Aspergillus***

Aspergillus is the most common cause of mycotic keratitis. *Aspergillus* produces a variety of toxic metabolites and cause many opportunistic and primary infections such as onchomycosis, sinusitis, cerebral aspergillosis, meningitis, endocarditis, myocarditis, pulmonary aspergillosis, osteomyelitis, otomycosis, keratitis, endopthamitis, cutaneous aspergillosis, hepatosplenic aspergillosis, aspergillus fungemia and disseminated aspergillosis may develop with the infection of *Aspergillus*. The genus *Aspergillus* was the first recognised etiologies of corneal infection and remains common isolates from oculomycoses (Hua *et al.*, 2010). The *Aspergilllus* are readily recognized by their morphology. The conidiophore with its swollen terminal end is surrounded by a flask shaped sterigmata, each of which produces long chains of conidia that radiate from the terminal end. Their hyphae are septated and branched dichotomously. The colour of the colony is different according to the species of *Aspergillus* such as *A. flavus* has yellow green surface with goldish to red brown reverse, *A. fumigatus* has blue-green to grey surface with white to tan reverse. *A. niger* has black surface with white to brown reverse and *A. terreus* has cinnamon to brown surface with white to brown reverse. These fungi produce mycotoxin and also act as allergen. They give positive complement test and immunodiffusion test. *A. fumigatus* is a thermotolerant fungus and grows well at the temperature over 40ºC. This property is unique to *A. fumigatus* among the *Aspergillus* species. *A. fumigatus* can grow at a temperature range of 20ºC to 50ºC. The first cases

of *A. tamari* fungal keratitis reported by Kredics *et al*, 2007 in Coimbatore, South India and they reported as fourth known case worldwide involving this unusual opportunistic human pathogen. It is able to produce several toxic secondary metabolites, including cyclopiazonic acid and fumigaclavines. It has rarely been encountered as a human pathogen. The only known cases are an eyelid infection, invasive nasosinusal aspergillosis in an immunocompetent patient, and onychomycosis (Larone, 2005; Prajna *et al*., 2008).

b. *Fusarium*

They are the widespread saprobic fungi that cause important diseases of plants and humans. They are found in normal mycoflora of commodities, such as rice, bean, soyabean, and other crops. *Fusarium* spp are causative agents of superficial and systemic infections in humans. Infections due to *Fusarium* spp are collectively referred to as fusariosis. Trauma is the major predisposing factor for the development of cutaneous infection due to *Fusarium* strains. The most virulent of *Fusarium* spp is *Fusarium solani*. *F. solani* can replicate at 35ºC and produces at least nine complex mycotoxins and destructive enzymes. Keratitis, endophthalmitis, otitis media, onchomycosis, cutaneous infections particularly of burn wounds, mycetoma, sinusitis, pulmonary infections, endocarditis, peritonitis, septic arthritis, disseminated infections, and fungemia due to *Fusarium* spp have been reported. *Fusarium* infections are difficult to treat and the invasive forms are often fatal. Macroscopically, the colour of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink or purple. From the reverse, it may be colourless, tan, red, dark, purple or brown. A sclerotium, which is the organised mass of hyphae that remains dormant during unfavorable conditions, may be observed macroscopically and is usually dark blue in colour. Hyaline septate hyphae, conidiophores, phialides, macroconidia, and microconidia are observed microscopically. Chalmydospores are also produced by some of the species of *Fusarium*. Phialides are cylindrical, with a small collarette, solitary or produced as a component of a complex branching system. Macroconidia are produced from phialides on unbranched or branched conidiophores. They are two or more celled, thick walled, smooth, and cylindrical or sickle shaped. Macroconidia have a distinct basal foot cell and pointed distal ends. Microconidia are formed on long or short simple conidiophores. They are 1-celled, smooth, hyaline, ovoid to cylindrical, and arranged in balls. Chlamydospores, when present, are sparse, in pairs,

clumps or chains. They are thick walled, hyaline, intercalary or terminal. Clusters of *Fusarium* keratitis were reported among contact lens users in Asia beginning in February 2006 (Bharathi *et al*., 2007). Outbreaks of nosocomial fusariosis have also been reported. Existence of *Fusarium* in hospital water distribution systems may result in disseminated fusariosis. *Fusarium* may also exit in soil of potted plants in hospitals. These plants constitute a very hazardous mycotic reservoir for nosocomial fusariosis (Prajna *et al*., 2008).

***c. Penicillium* spp**

Penicillium spp are cosmopolitan and predominant in temperate climate. They are usually a contaminant or a secondary invader. The colony grows moderately rapid to rapid. The colony has velvety to powdery and green, blue green, grey green, white, yellow or pinkish colour on the surface and reverse is usually pale to yellowish, rough walled red or brown. *Penicillium* has septate hyaline hyphae, simple or branched conidiophores, phialides form the brush-like clusters at the tips of the conidiophores. The conidia are round, unicellular, and visualized as unbranching chains at the tips of the phialides. Pulmonary infections, keratomycosis, onchomycosis, external ear infections, cutaneous lesions, bladder infections and endocarditis are the infections caused due to *Penicillum* spp.

d. Acremonium (Cephalosporium)

Acremonium is cosmopolitanin in nature, isolated from soil and plant debris. It is a rare cause of keratitis and endophthalmitis. The growth rate of the colonies is moderately rapid, maturing within 5 days. The texture of the colony is compact, flat or folded, and occasionally raised in the centre. It is glabrous, velvety, and membrane-like at the beginning. The surface of the colony becomes cottony due to the overgrowth of loose hyphae. The colour of the colony is white, pale pink or pale grey on the surface. The reverse side is either uncoloured or a pink. These fungi possess hyaline, septate hyphae which are very fine and narrow. The phialides are separated from hyphae by a septum and taper towards their apices. Conidia are at the apices of the phialides which appear in clusters, in balls or rarely as fragile chains. They may be single or multicellular, fusiform with a slight curve or resemble a shallow crescent. The grains (500-2000µm in size) of *Acremonium* spp are regular and oval to round in shape. When stained with H&E, poorly staining dense hyphal packs are observed (Smith and Charles, 1990).

2. Pigmented or Dematiaceous fungi

Dematiaceous fungi are characterized by the development of a brown to olive to black colour in the cell walls of their vegetative cells, conidia, or both, which results in pigmented colonies on culture. These ubiquitous, cosmopolitan, and opportunistic pathogens are normally associated with soil or plants but have been isolated from various human infections including keratitis (Berger *et al*., 1991). Some of the pigmented and septated filamentous fungi are described below:

a. Cladosporium

They are the most common dematiaceous fungi to cause keratitis and endophthalmitis. Many species are cosmopolitan fungi or found on soil, plant debris and leaf surfaces. Mostly *Cladosporium* are isolated from humid air. Their growth rates are moderate at 25ºC on SDA and have velvety to powdery texture, green to black colour from the front and black colour from the reverse. They produce septate brown hyphae, erect and pigmented conidiophores and conidia.

b. Curvularia

They are the most common dematiaceous fungi to cause corneal ulcer (Garg *et al*., 2000). Other ocular infections consist of conjunctivitis, dacryocytitis, sino-orbital cellulites, and endopthalmitis however; the cornea is the most commonly infected site. Most species are facultative pathogens of tropical or subtropical plants. They produce rapidly growing, woolly colonies on Potato Dextrose Agar at 25ºC. The colour of the colony is white to pinkish gray initially and turns to olive brown or black as the colony matures from the front whereas it is dark brown to black from the reverse. They have septated, brown hyphae, brown conidiophores, and conidia. Conidiophores are simple or branched and are bent at the points where the conidia originate. This bending pattern is called sympodial geniculate growth. The conidia are straight or pyriform, brown, multiseptated, and have dark basal protuberant hilla. The swelling of the central cell usually gives the conidium a curved appearance. The number of the septa, shape (straight or curved), colour of the conidia (dark vs pale brown), as well as the existence of dark median septum, and the prominence of geniculate growth pattern are the major microscopic features that help in differentiation of *Curvularia* spp among each other.

c. Bipolaris

Bipolaris is common cause of keratitis and endophthalmitis. It is cosmopolitan, although some species are mainly found in tropical or subtropical areas. They are saprobes or pathogens of numerous species of plants. *Bipolaris* colonies grow rapidly and become mature within 5 days. The texture is velvety to woolly. The surface of the colony is initially white to grayish brown and becomes olive green to black with a raised grayish brown and becomes olive green to black with a raised grayish periphery as it matures. The reverse is also darkly pigmented and olive to black in colour. The hyphae are septated and brown. Conidiophores are brown, simple or branched, geniculate or sympodial, bending at the points where each conidium arises. This property leads to the zigzag appearance of the conidiophore. The ascopores are flagelliform or filiform, hyaline in nature and are found in clavate-shaped or cylindrical asci. Each ascus contains eight ascospores. The hyphae may be branched or constricted at the points of septation. *Bipolaris* can infect both immunocompetent and immunocompromised host (Shah and Das, 2005).

d. Alternaria

Alternaria is also a cosmopolitan dematiaceous fungus commonly isolated from plants, soil, food, and indoor environment. It is the occasional agents of keratitis. Other infections include onychomycosis, ulcerated cutaneous infection, chronic sinusitis and rare cases of deep infection have been reported in immunocompromised patients. *Alternaria* grows rapidly and matures within 7 days. The colony is flat, downy to woolly and is covered by grayish, short, aerial hyphae in time. The surface is grayish white at the beginning which later darkens and becomes greenish black or olive brown with a light border. The reverse side is brown to black due to melanin-like pigment production. *Alternaria* has septated, brown hyphae. Conidiophores are also septate and brown in colour, sometimes producing a zigzag appearance. They bear simple or branched large conidia which may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid to obcalvate, darkly pigmented, muriform, and smooth or roughened. The end of the conidium nearest the conidiophores is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia.

3. Non-septated fungi

a. Rhizopus

Rhizopus is non-septated fungi of ocular importance. It is cosmopolitan and frequently isolated from soil and agricultural products. Some species are plant pathogens. The spores which are infective are found in air and dust. The colonies of *Rhizopus* grow very rapidly and mature within 4 days. The texture is cotton-candy like. The colour of the colony is white and turns grey to yellowish brown in time and reverse is white to pale. It is a common laboratory contaminant. It reproduces both sexually and asexually by zygospores and sporangiospores respectively. It has broad, branched nonseptated hyphae. Rhizoids are located at the point where the stolons and sporangiophores meet.

2.7.1.2 Yeasts

Yeasts consist primarily of *candida species. Candida* produces white to creamy, soft, opaque, pasty, smooth to wrinkled colonies on culture media. *Candida* is an extreme opportunistic organism and mostly affects the immunocompromised individuals. It is the most common ocular fungal pathogen, because it is also implicated in diseases of the eyelids, conjunctiva, lacrimal drainage system and retina. *Candida albicans* is the species most frequently identified, however *Candida parapsilosis* and *Candida tropicalis* are also seen. Eighty percent of eye infection is caused by *C. albicans*. It is a saprophytic found in soil, water and food. It is a commensal of all the mucous membranes of the human body and skin including conjunctiva. *C. albicans* can transform from a blastoconidial to a pseudohyphal phase under appropriate conditions. As with filamentous fungi, the large size of the pseudohyphae precludes complete ingestion by neutrophils. It is a gram positive organism and stains darker than cocci. The organism stains with Gram's stain, Giemsa stain, Gomori's Methenamine Silver stain and periodic acid-schiff stain. With the Giemsa Stain the yeasts cells absorb the stain and appear purple or blue. It produces two types of spores, the chlamydospores and blastospores. This unicellular oval organism reproduces by budding and form pseudohyphae under reduced oxygen tension or in tissue. The pseudohyphal phase is the most invasive and virulent phase, are not parallel to one another and have constrictions. Terminal chlamydoconidia may be formed with extended incubation. *C. albicans* is also a predominate species in fungal biofilms on medical devices (Prajna *et al*., 2008).

C. albicans is identified by germ tube test method. It is the gold standard method for the identification of *C. albicans.* In this method, a yeast colony is inoculated into 0.5 ml of human serum in a small test tube and incubated at 37ºC for 3-4 hours. After the

incubation, a drop of the serum yeast culture is transferred to a glass slide and is covered with a cover slip avoiding air bubbles. The preparation is examined on microscope for sprouting yeast cells that is tube like outgrowths from the cells known as germ tubes using the 10X and 40X objectives (Collee *et al*., 1996).

The examination of fungal elements microscopically is important because it provides the physician with a rapid diagnosis and information regarding the possible need for the treatment and is also helpful in determining the significance of the organisms that will later be definitely identified on culture (Larone, 2005).

Table 1: Identification of the most common species of *Aspergillus*

Species	**Pathogenicity**	**Macroscopic morphology**	**Microscopic morphology of conidiophore**	**Microscopic morphology of phialdies**
Aspergillus fumigatus	Most common cause of aspergillosis	Velvety or powdery at first white, then turning dark greenish to grey, reverse white to tan	Short (<300µm), smooth	Uniseriate, usually only on upper two- thirds of vesicle, parallel to axis of conidiophore
Aspergillus niger	Usually considered a contaminant, but also known to cause disease in the debilated	Woolly at first white to yellow, then turning black, reverse white to yellow	Long smooth	Biseriate, cover entire vesicle, form "radiate" head.
Aspergillus flavus	Usually a contaminant but also known to cause disease, commonly associated with aflatoxins	Velvety, yellow to green or brown, reverse goldish to red brown	Variable length, rough, pitted, spiny	Uniseriate and biseriate, cover entire vesicle, point out in all directions
Aspergillus terreus	Usually a contaminant but also known to cause infection	Usually velvety, cinnamon brown, reverse white to brown	Short (<250µm), smooth	Biseriate, columnar (round hyaline cells produced on mycelium submerged in agar)

Species	Pathogenicity	Macroscopic morphology	Microscopic morphology of conidiophore	Microscopic morphology of phialdies
Aspergillus versicolor	Usually a contaminant but also known to cause diseases	Velvety, at first white, then yellow, occasionally pinkish, reverse white or yellow, orange or red	Long, smooth	Biseriate, loosely radiate, cover most of vesicle (hulle cells may be present)
Aspergillus nidulans	Usually a contaminant but has been involved in infection.	Velvety, usually green, but buff to yellow where cleistothecia form, reverse purplish red becoming dark	Short (<250µm), smooth, brown	Biseriate, short columnar cleistothecia usually present with reddish ascospores, hulle cells often abundant
Aspergillus clavatus	Commonly considered as contaminant	Feltlike, blue green, reverse white, may become brown with age	Long, smooth	Uniseriate, closely crowded on huge clavate vesicle (approx 200×40µm)

The organisms which are causing the ulceration of the cornea, may gain access to the interior of the eye as a result of perforation and cause purulent iridocystics, endophthalmitis and even panophthalmitis (Nema and Nema, 1998). The cornea does not generally have any colonizing bacteria. Bacteria from eyelids may contaminate the ocular surface and cause corneal ulcer. Pathogenic bacteria consist of virulence factors which are responsible for infectious keratitis. These include specific antigens, proteolytic enzymes, haemolysins and toxins. *Nocardia* are also associated with infections after trauma, contact lens wear and following laser in situ keratomileusis (LASIK) surgery (Verma and Tuft, 2002). The pathogenic species are *Nocardia asteroids* and *Nocardia brasiliensis*. The virus can also infect individual layers of the cornea or in more severe form it may involve all the layers of cornea. Herpes simplex virus, varicella zoster virus and adenovirus are involved in keratitis (wong *et al*., 2003). Keratitis can occur due to various protozoa, of which *Acanthamoeba* is the

most notorious (Awward *et al*., 1986). Other protozoa which has been increasingly isolated from cases of cornea ulcer includes *Microsporidia* (Sharma and Vajpayee, 2008).

2.8 Antibiotics used to treat fungal infection

Antifungal agents used in mycotic keratitis include the polyene and azole compounds. The polyenes include natamycin and amphotericin B. Polyenes disrupt the cell by binding to fungal cell wall ergosterol forming a polyenesterol complex that alters membrane permeability, depleting essential cellular constituents and are effective against both filamentous and yeast forms. Natamycin, available as 5% suspension, is considered the drug of choice for filamentous fungi (O'Day, 1987). However, a prospective study comparing the efficiency of natamycin 5% and econazole 2% reported that there is no statistically significant difference between the two drugs. Natamycin binds preferentially to ergosterol on the fungal plasma membrane and causes localised membrane disruptions by altering membrane permeability.

Moreover, natamycin is expensive; the supply is erratic and often times difficult to obtain. It is derived from *Streptomyces natalensis*.

Amphotericin B is derived from a strain of *Streptomyces nodosus*. It can be fungistatic or fungicidal and is available as a systemic preparation. It can also be used through the subconjunctival, intracameral, intravitreal and intravenous routes. The penetration of topically applied amphotericin B is poor in cornea with intact epithelium. Topical application may cause punctuate epithelial erosions. The spectrum of activity of amphotericin B covers *Candida* spp and *Aspergillus* spp. It is not effective against *Fusarium* species (O'Day, 1987).

The azole compounds include triazoles: econazole, clotrimazole and the imidazoles: fluconazole, itraconazole and voriconazole. Azoles inhibit ergosterol synthesis at low concentrations, and appear to cause direct damage to cell walls at higher concentrations. Econazole and clotrimazole are available as 1 % topical preparation eye drops and in ointment form. The imidazoles exhibit antifungal activity through two mechanisms of action depending on the concentration. At lower concentrations, they are fungistatic and inhibit sterol-4-alpha demethylase, a microsomal P450 related enzyme, while they are fungicidal at higher concentrations causing direct membrane damage to the phospholipids present in the fungal cell wall. It is found that the effect of econazole is equal to that of natamycin (Prajna *et al.,* 2003) whereas it is suggested

that clotrimazole as monotherapy is not an ideal choice (Mselle, 1999). It is possible that use of both natamycin and econazole in the management of fungal keratitis may improve chances for more rapid resolution of corneal ulcers, especially since the mechanisms of action are different. However, further studies are required to validate this hypothesis (Prajna *et al.*, 2003). The imidazoles; miconazole and ketoconazole can be used systemically for keratomycosis due to their relatively reduced systemic toxicity and better corneal penetration. Oral fluconazole and ketoconazole are absorbed systemically with good levels in the anterior chamber and the cornea. Therefore, they should be considered in the management of deep fungal keratitis (Thomas, 2003b). Fluconazole is a fungistatic bitriazole and is considered as a topical and systemic agent in the treatment of fungal keratitis due to *Candida* and *Aspergillus* spp (Avunduk *et al.*, 2003; Rao *et al*., 1997). Voriconazole is a new azole antifungal agent derived from fluconazole and shows a broder spectrum of activity against *Candida, Aspergillus, Scedosporium, Fusarium* and *Paecilomyces*. As with other triazole antifungal agents, voriconazole exerts its effect primarily through inhibition of cytrochrome P450- dependent 14 – sterol demethylase, an enzyme responsible for the conversion of lanosterol to 14- *demethyl* lanosterol in the ergosterol biosynthetic pathway. Voriconazole is as efficacious as amphotericin B for the treatment of invasive aspergillosis. Echinocandins are used for systemic mycoses as the new antifungal drugs. The target of the Echinocandins is the synthetic cell wall enzyme complex β-1, 3-D glucon synthase. The antifungal spectrum is restricted to *Candida* species and *Aspergillus* species and is not active against *Fusarium* species (Srinivasan, 2004).

2.8.1 Surgical therapy

Frequent corneal debridement with a spatula is helpful as it helps to reduce the bulk of the fungal organisms and enhance penetration of the topical antifungal agent. The use of N- *butyl* cyanoacrylate tissue adhesive in the management of corneal thinning or perforation associated with active fungal keratitis has been reported (Garg *et al*., 2004). Approximately, one third of fungal infection fails to respond to medical treatment and may result in corneal perforation. A therapeutic penetrating keratoplasty is necessary in these cases. Topical antifungal therapy in addition to systemic fluconazole or ketoconazole should be continued following penetrating

keratoplasty (Dong and Shi, 2001). Subconjunctival injections of amphotericin B are useful as an adjunctive treatment of advanced fungal keratitis. This treatment assures compliance and may be an alternative to avoid a surgical intervention in an acute stage of advanced fungal keratitis (Carrasco and Genesoni, 2011).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials requirements

All the materials required during the study are listed in the Appendix II.

3.2 Methods

The study was conducted from July 2010 to February 2011 in Tilganga Institute of Ophthalmology, Gaushala, Kathmandu. Altogether 300 suspected cases of corneal ulcers were included in this study. The study type was cross-sectional.

3.2.1 Collection of samples

The sample was collected from the suspected patients attending Out Patient Department (OPD) of Tilganga Institute of Ophthalmology, Gaushala, Kathmandu. Corneal scraping was performed under aseptic condition by an ophthalmologist using a sterile Kimura spatula after the application of 5% xylocaine as an anesthetic agent. Scraped materials were taken from the edges and base of the ulcer.

3.2.2 Laboratory settings

The samples were processed in the microbiology laboratory of Tilganga Institute of Ophthalmology.

3.2.3 Data collection

During the collection of sample from suspected cases, questionnaire was filled which included name, age, address, gender, occupation, staining results, culture reports, identification of organisms etc.

3.3 Sample processing

3.3.1Microscopic examination

Materials obtained from scraping were smeared onto three slides to prepare 10% KOH wet mount or Lactophenol Cotton Blue (LPCB) stain, Gram's stain and Giemsa stain. These slides were prepared and observed under the microscope. The procedures of the preparation and staining are described in the appendix-III.

3.3.2 Culture

The materials obtained from scraping were streaked directly onto the surface of Sabouraud's Dextrose Agar (SDA), Blood Agar (BA) and Chocolate Agar (CA) in "C" shaped and was also inoculated in Thioglycolate broth. The inoculated media except for CA were incubated at 27ºC in aerobic condition for 7 days to 28 days; however length of incubation varied depending on the specimen and or culture. CA was incubated in a CO_2 incubator.

3.3.2.1 Processing of culture

Fungal plates were observed daily for the first week, then twice a week up to 4 weeks and if there were no growth, the plates were discarded.

3.3.3 Identification of fungal isolates

3.3.3.1 Macroscopic observation

The filamentous fungal hyphae were identified by their colonial characteristics such as colour, texture, colour of the reverse (underside) of the colony, diffusible pigments, growth zones, aerial and submerged hyphae, growth rate, colony topography etc.

3.3.3.2 Microscopic observation

a. Direct examination

In the direct examination, the fungal hyphae, conodial head, conidiophores, terminal

vesicles, macroconidia, microconidia, chlamydospores, phialides, sporangiospores, rhizoids, ascus, ascospores, budding, capsule etc were observed.

1. A portion of the colony was directly picked up from the plate with the help of sterile forcep and was placed in a slide.
2. A drop of Lactophenol Cotton Blue was placed onto that portion, was covered with coverslip avoiding air bubbles and was pressed gently to flatten the preparation.
3. Then the slide was directly observed under microscope at 10X and 40X respectively.

b. Adhesive tape technique

The aerial hyphae and spores were examined microscopically by Adhesive Tape Technique.

1. A drop of Lactophenol Cotton Blue was placed at the centre of a clean grease free slide.
2. A piece of adhesive cellulose tape was cut and was folded back on itself with the adhesive side turned outward.
3. The adhesive side of the tape was pressed into the surface of the colony and was pulled away. The aerial hyphae of the colony were remained glued on to the tape surface.
4. The tape was placed in a drop of Lactophenol Cotton Blue on the slide avoiding air bubbles.
5. The slide was examined microscopically at 10X and 40X objectives respectively (Larone, 2005; Prajna *et al*., 2008).

c. Germ tube test

For *C. albicans* identification was done by the following methods.

1. 0.5ml of human serum was pipetted out into small test tube.
2. A yeast colony was inoculated into the serum using a sterile loop and the test tube was incubated at 37ºC for 3-4 hours.
3. After the incubation, a drop of the serum yeast culture was transferred to a glass slide and was covered with a cover slip avoiding air bubbles.
4. The preparation was examined for sprouting yeast cells that was tube like outgrowths from the cells known as germ tubes using the 10X and 40X objectives.

3.4 Quality control

Quality of each test was maintained by using standard procedures. One representative sample of each lot of the prepared culture plate was incubated at 25-30ºC for 4 days to check the sterility of the media.

3.5 Data analysis

Statistical analysis was done using SPSS version 16.

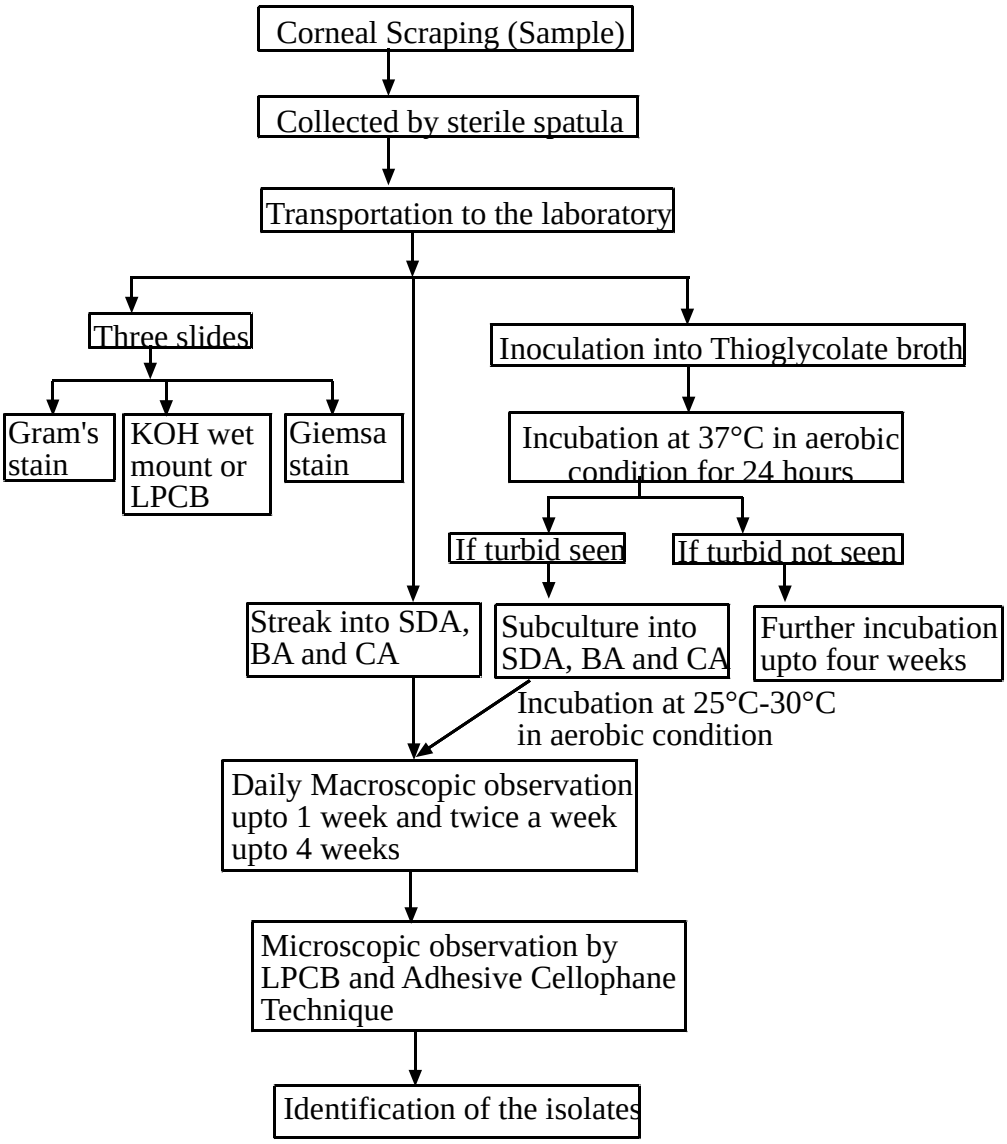

Fig: 1 Flow chart for identification of fungal pathogens in corneal ulcers

CHAPTER IV
RESULTS

A total of 300 corneal scrapings were collected from the corneal ulcer suspected patients visiting TIO from July 2010 to February 2011. Out of 300 samples collected, 21% (n=63) samples had shown growth of fungal pathogens. Out of 63 fungal isolates, majority of the isolates i.e. 38.1% (n=24) were identified as *Aspergillus* spp followed by *Fusarium* spp 19% (n=12) and *Curvularia* spp 14.3% (n=9).

4.1 Pattern of sample distribution

Among 300 samples of corneal ulcer collected the pure growth of fungal organisms was observed only on 19.7% (n=59) samples and 1.3% (n=4) samples indicated mixed fungal growth with other microorganisms.

Figure 2: **Distribution pattern of fungal growth**

Single growth of organisms was higher than mixed growth. Single to mixed growth ratio was found to be 14.7:1.

4.2 Genderwise distribution of isolated organisms

Among the total number of 300 suspected samples, 52.7% (n=158) samples were collected from male patients and 47.3% (n=142) samples were collected from female patients. And also, more number of fungi was isolated from male patients as compared to the female patients. The mean age calculated was 44.8 years in male and 41.7 years in female. Also, in this study number of male patients and that of female gave a ratio of 1.1:1.

Table 2: Gender wise distribution of organism isolated from total samples

Gender	No. of samples collected	Organisms isolated (%)	P value
Male	158	43 (27.1)	0.157
Female	142	20 (14.1)	
Total	**300**	**63 (21)**	

Out of the total 300 corneal scraping samples, 27.2% (n=43) organisms were isolated from the male patients whereas 14.1% (n=20) organisms were isolated from female patients. The total organisms isolated were 21% (n= 63). Higher rate of infection was seen in males than females. However, there was no significant difference between the males and females (p=0.157).

4.3 Agewise distribution of isolated organisms

On the basis of age group, there was uneven distribution of cases of corneal ulcers. The working age group was mostly infected. However, the incidence of infection was low in the extremes of ages. And also, male patients were more infected than female patients.

Table 3: Age wise distribution of organism isolated from total samples

Age group	Organisms from		Total	P value
	Male (%)	Female (%)		
below 20	4 (6.3)	1 (1.6)	5 (7.9%)	0.199
21-30	7 (11.1)	5 (7.9)	12 (19%)	
31-40	13 (20.6)	2 (3.2)	15 (23.8%)	
41-50	5 (7.9)	6 (9.5)	11 (17.5%)	
51-60	9 (14.3)	5 (7.9)	14 (22.2%)	
more than 60	5 (7.9)	1 (1.6)	6 (9.5%)	

Total	**43 (68.2)**	**20 (31.7)**	**63 (100)**	

Out of 63 total isolated fungi in this study, we found that the highest number of fungi was isolated from 31-40 (23.8%) age group, then followed by 51-60 (22.2%) age group.The maximum number of fungi (20.6%) was isolated from male patients between the ages 31 and 40. Similarly, more fungi were isolated in 41-50 (9.5%) age group from female patients. However, there was no significant difference between the organisms isolated from male and female patients (p= 0.199).

4.4 Monthwise distribution of the corneal ulcer cases

The corneal ulcer cases were unevenly distributed during the study period. It showed seasonal variation i.e. more infected cases were seen in early winter season where as there were least infected cases in summer season.

Figure 3: Month wise distribution of the corneal ulcer cases

The corneal ulcer cases were highest (28.6%) during the month of November and December followed by the month of October (14.3%) and least (1.6%) in February.

4.5 Occupationwise distribution of corneal ulcer cases

The patients were categorized according to their occupation in which majority of cases were from agriculture related workers, housewives and environmental exposure groups.

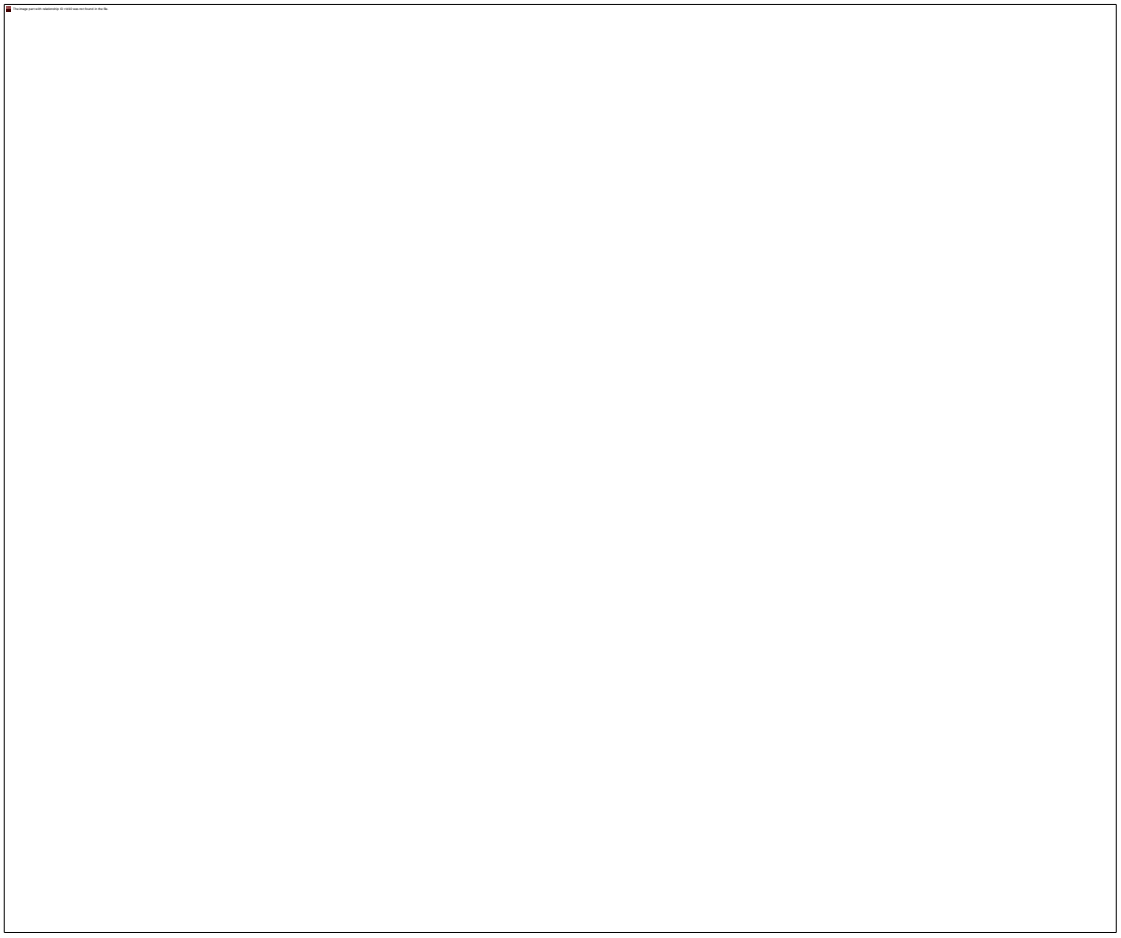

Figure 4: Occupation wise distribution of corneal ulcer cases

Agricultural workers (38.1%) were the most infected cases followed by housewives (22.2%). Students were least (3.2%) infected.

4.6 Comparison of KOH wet mount preparation and positive fungal isolates

Most of the positive KOH wet mount tests had shown growth of organisms. However, all the positive culture tests had not given positive KOH wet mount tests.

Table 4: Comparison of KOH wet mount preparation and fungal culture

S.N.	Observation	No. of cases	Percentage
1	Both KOH wet mount and growth positive	56	88.9%
2	KOH wet mount negative and growth positive	7	11.1%
	Total	**63**	**100%**

Out of 63 cases of fungal corneal ulcers, 88.9% (n=56) of KOH positive slides showed growth of organisms. Similarly, 11.1% (n=7) positive growth of organisms showed KOH negative tests.

4.7 Distribution pattern of fungal isolates

Altogether, 63 fungi of nine different genus were isolated from 300 suspected corneal ulcer cases. Out of 63 fungal isolates, 68.25% (n=43) were isolated from male patients and 31.75% (n=20) were isolated from female patients.

Table 5: Distribution pattern of fungal isolates

S. N.	Organisms isolated	Male (%)	Female (%)	Total (%)
1.	*Aspergillus* spp	15 (23.8)	9 (14.3)	24 (38.1)
2.	*Acremonium* spp	2(3.2)	1(1.6)	3 (4.8)
3.	*Candida albicans*	0 (0)	2 (3.2)	2 (3.2)

4.	*Fusarium* spp	10(15.9)	2(3.2)	12(19)
5.	*Cladosporium* spp	3(4.8)	2(3.2)	5(7.9)
6.	*Penicillium* spp	4(6.3)	1(1.6)	5 (7.9)
7.	*Rhizopus* spp	1(1.6)	0 (0)	1 (1.6)
8.	*Curvularia* spp	7(11.1)	2(3.2)	9 (14.3)
9.	*Bipolaris* spp	1(1.6)	1 (1.6)	2 (3.2)
Total		**43(68.2)**	**20(31.7)**	**63 (100)**

The majority of the fungal isolates were *Aspergillus* 38.1% (n=24) followed by *Fusarium* 19% (n=12) and *Curvularia* 14.3% (n=9). In female patients, *Aspergillus* was the most commonly isolated where as *Aspergillus*, *Fusarium*, *Curvularia* were common isolates in male patients.

4.8 Pattern of different species of *Aspergillus* isolated

Aspergillus was the most commonly isolated fungi. Altogether, 24 *Aspergillus* of four different species were identified. There were 62.5% (n=15) isolates from male patients and 37.5% (n=9) isolates from female patients.

Table 6: Pattern of different species of *Aspergillus* isolated

Organisms isolated	**Organisms from**		**Total**	**p value**
	Male (%)	**Female (%)**		
Aspergillus fumigatus	9 (37.5)	6 (25)	15 (62.5)	0.083
Aspergillus flavus	3 (12.5)	2 (8.3)	5 (20.8)	
Aspergillus terreus	2 (8.3)	0 (0)	2 (8.3)	
Aspergillus tamari	1 (4.2)	1 (1.2)	2 (8.3)	
Total	**15(62.5%)**	**9(37.5%)**	**24**	

Aspergillus fumigatus 37.5% (n=9) in male and 25% (n=6) in female were the most frequently isolated species of *Aspergillus. Aspergillus terreus* and *Aspergillus tamari* 8.3% (n=2) were the least commonly isolated. There was no significant growth of *Aspergillus fumigatus* among all isolates (p=0.083).

PHOTOGRAPHS

Photograph 1: *Fusarium* species on Sabouraud Dextrose Agar (Reverse side)

Photograph 2: *Aspergillus* species on Sabouraud Dextrose Agar

Photograph 3: *Fusarium* species on Blood Agar

Photograph 4: *Curvularia* species on Sabouraud Dextrose Agar

CHAPTER V
DISCUSSION

Corneal ulcer is a leading cause of ocular morbidity and blindness worldwide including Nepal. It is defined as a loss of epithelial tissue from the surface of the cornea due to progressive erosion and necrosis of the tissue. It generally results from bacterial, protozoal, viral or fungal infection. However, the spectrum of etiologic agents varies based on geographic and socio-economic differences across and even within countries. Studies from western centre report bacteria to be the etiologic agent in 70% to 91% of culture-positive corneal ulcers (Keay *et al.*, 2006). However, Africa and India have shown a higher burden of fungal organisms, between 35% and 72% of culture-positive ulcers (Leck *et al*., 2002). The prior studies of corneal ulcers in Nepal showed the incidence of fungal ulcers at between 21% and 24% (Upadhyay *et al*., 1982 and 1991) and 36% from the western periphery of Nepal (Ganguly *et al*., 2011). Fungal keratitis is less common than bacterial keratitis and more devasting. Fungi can penetrate deep into the stroma and through an intact descemet membrane, gaining access to the anterior chamber. A large number of dematiaceous fungi, filamentous fungi, yeasts and other dimorphic organisms have been implicated as aetiological agents for fungal keratitis worldwide (Chitra *et al*., 2006).

The present study conducted at the microbiological laboratory of Tilganga Institute of Ophthalmology showed that fungal corneal ulcer is being the common problem in our country. Out of total number of 300 corneal samples processed under study, 21% of the culture was fungal positive which was 39.1% of total culture positive growth. This result was similar to the results obtained by Gopinathan *et al*., 2009 (35%), Panda *et al*., 2007 (38.2%), Sirikul *et al*., 2008 and Laspina *et al*., 2004 (26%). About 21% (N=63) fungi of nine different genus were isolated. A study conducted by Srinivasan, 2004 reported fungal keratitis as an enormous public health problem in south India. Fungi, especially *Fusarium* (47%) and *Aspergillus* (16%), were identified as the aetiological agents responsible for 44% of all corneal ulcers in that study. Reports from Ghana and Northern Tanzania have reported fungi as the aetiological agent in over 50% of culture positive cases of keratitis (Hagan *et al*., 1995; Poole *et al*., 2002).

The infected cases in this study were categorized according to the age wise and gender wise. The age and sex of the host is not a direct risk factor in this disease as

infection rates are comparable if the nature and degree of exposure are equivalent. According to the distribution pattern, the vast majority of patients were of working age. Low incidence in patients at extremes of ages is attributable to their minimal involvement in the field work. Fewer children are involved in agricultural works, so the agricultural injury did not commonly predispose to corneal ulceration in children rather non-agricultural trauma may account for corneal ulcer. Malnutrition, xerophthalmia and measles are the predisposing factors for the incidence of corneal ulcer in children (Upadhyaya *et al*., 1991). The increasing incidence of this infection with age is probably a function of an increased opportunity for exposure. The highest incidence between the ages of 21-60 is due to their greater involvement in their agricultural fields, industries, factories, use of contact lens, corticosteroids and other occupational hazards.

The sex wise distribution of infected cases was also determined. The present study reveals a male predominance of fungal corneal infection with a number of male patients being 43 (68.3%) out of 158 male patients and that of female were 20 (31.7%) out of 142 female patients giving a ratio of 2.1:1. This was similar to other studies done by Lavaju *et al*., 2009; Gonzales *et al*., 1996 and Liesegang and Forster., 1980. This may be due to the greater involvement of males in outdoor activities than females.

The present study indicated that corneal ulcers were encountered in all ages. The highest number of organisms 20.6% (n=13) was isolated from the age group 31-40 and followed by the age group 51-60 by 14.3% (n=9) in male patients whereas 9.5% (n= 6) organisms were isolated from age group 41-50 in female patients as the maximum number which was followed by the age group 51-60 and 21-30 by 7.9% (n=5). This observation correlates well with the study done by Ganguly *et al*., 2011 where the prevalence was highest (28%) among patients between 31 to 40 years and with the study done by Chowdhary and Singh, 2005 where 37% of cases of mycotic ulcer involved the same age group 31 to 40 years.

Agricultural workers were found to be the most affected by corneal ulcer followed by the housewives. This is due to the fact that as farmers work in the fields they are more prone to ocular trauma which is the most frequent predisposing factor. The high prevalence among housewives may be explained by the involvement of women in agricultural field activities and inability of most women to access medical services. This fact correlates well with a previous study done in Nepal by Upadhyay *et al*.,

1988, where 49.6% of affected patients were farmers and 22.5% were housewives compared to the 38.1% and 22.2% in the respective groups in this study. Trauma remained an important predisposing factor in all age groups affecting children, adults and elderly in varying proportions (Bharathi *et al*., 2003). Most injuries occurring in children are non-agricultural while in adult life and later they are very often agriculture induced. Domestic or recreational injuries are the prime cause in children (Cruz *et al*., 1993). Vegetative matters are the most frequently identified traumatic agents. Corneal ulcers are common in people who wear contact lenses especially if they wear them overnight. Filamentous fungi are more commonly associated with cosmetic lens wear, and yeasts are more frequently associated with therapeutic lens use (Jurkunas *et al.*, 2009).

The frequency of mycotic ulcer was found to be higher in the months of November and December. In the most part of Nepal, the harvesting period of agricultural products mainly paddy is the months of October to December. Chowdhary and Singh, 2005, has also documented an increased incidence of mycotic ulcer in the months of September and October. However, this fact was not similar to the study done by Ganguly *et al*., 2011 where no significant seasonal variation in incidence of fungus ulcer was noted. As paddy is the principal crop in Nepal, it is the main agent of injury causing fungal corneal ulcer. In this study also people are mostly infected in these months. Besides, these months have higher humidity because they are the months of early winter. In the developing countries where an agricultural environment and humid climate couple with malnutrition, all are conducive to the development of infective keratitis from minor trauma (Whitcher and Srinivasan, 1997). In addition, fungi are normally present as commensals in conjunctival flora of healthy eyes in about in 3-28% and can invade the cornea in suitable conditions such as following trauma, corticosteroid administration, or in diseased corneas. Trauma and corneal ulceration is largely a cause of monocular blindness although it is an important cause of bilateral blindness as well (Whitcher *et al*., 2001). Corneal injuries have been reported to be associated with various insects and spiders (Gilboa *et al*., 1997).

The eye is being small organ; the specimens recovered from them to isolate the organisms are very small that makes recovery and identification of organisms difficult. Also, the specimens do not withstand transport time. Demonstration of fungal elements in direct smear examination has been difficult. So Gram's stain, Giemsa stain were also evaluated for the demonstration of fungi in direct smear.

Direct microscopic evaluation is the most valuable and rapid diagnostic tool for the detection of fungal filaments in corneal scrapings. Giemsa stain and Gram stain are equally sensitive in detecting fungal elements (Jones, 1979). Thomas, 2003b has highlighted the sensitivity of various stains. Gram stain will identify fungal species in 45 to 73% of cases, and Giemsa will identify fungi in 66%. Lactophenol cotton blue has a sensitivity of 70 to 80%, Grocott methenamine silver staining of as much as 89% and calcofluor white of 80 to 90%. A retrospective study (Bharathi *et al*., 2003) of corneal ulcers reported that the sensitivity of the 10% KOH wet mount was higher (99.23%) than that of the Gram stained smear (88.73%) in the detection of fungal keratitis. 10% KOH wet mount is simple, cheap, rapid, and easy to interpret. It is an ideal method for practice in tropical and developing countries. The failure to detect fungal elements in some cases emphasizes the need for developing staining techniques for easy and quick visualization of fungi in direct smear. Polymerase chain reaction (PCR) and confocal microscopy are being used as new rapid diagnostic methods; they are not available in areas where fungal keratitis is highly prevalent (Srinivasan, 2003).

In this study filamentous fungi were the predominant cause of fungal corneal ulcers. The predominant fungi were genus *Aspergillus* (38.1%) which was followed by genus *Fusarium* (19%) and genus *Curvularia* (14.3%) respectively. This result was similar to the result obtained by Upadhyay *et al*., 1991. In another study by Chowdhary and Singh, 2005, *Aspergillus* species were found to be the most common fungal isolate accounting for 41% of cases. Among them, *Aspergillus niger* was the most common species isolated. In that study, *Fusarium* species were detected in 12.5% of cases which is lower than in this study. However, according to the report of (Ganguly *et al*., 2011) the spectrum of fungi isolated in fungal corneal ulcer were *Fusarium* species (31.9%), followed by unidentified dematiaceous (22%) and unidentified hyaline (16.1%). The marked virulence of *Fusarium* for the cornea is that it produces substances that help it to penetrate or toxins that help to produce the lesions (Sharma *et al*., 1993). Also, the ocular virulence of *Fusarium* may be due to its capacity for angioinvasion, which occuldes the intraocular vascular channels with secondary infection, haemorrhage, and necrosis (Guarro *et al*., 2003). *Candida albicans* was the most commonly isolated organism (45.8%), followed by *Fusarium* spp (25%) in (Tanure *et al*., 2000) report. A report from a rural region in north China, *Fusarium* was isolated from 73.5% of ulcers, with yeast isolated from only 2% of ulcers (Xie *et*

al., 2006). Prior ocular surface disease or penetrating keratoplasty was present in 97.4% patients with *Candida* infection, and 74.4% patients with *Candida* infection were using topical steroid at the time of diagnosis (Galarreta *et al*., 2007).

All of the fungi were grown within 15 days either in SDA, BA or CA or Thioglycollate broth. SDA is a readily available universal non-selective medium for the primary isolation of opportunistic fungi. So it is also the medium of choice for the primary isolation of ocular fungi. Fungal keratitis can be reliably confirmed on BA or CA, which support growth of both bacteria and fungus (Das *et al.*, 2010). In view of the fact various species of the genus *Aspergillus* are the commonest isolates in the part of the world; SDA may be used as primary isolation media for ocular fungi. However since SDA is available in most laboratories, it should be continued as medium of choice for fungus isolation (Das *et al.*, 2010).

Early recognition of fungal keratitis is important, but fungal infection is a diagnostic challenge (Vajpayee *et al.,* 1993). Specific treatment requires quick and accurate identification of the causative micro-organisms (Leck *et al.,* 2002). Large sized ulcers yielded fungi more frequently. Deep infection can be very hard to eradicate. A survey of community ophthalmologists in southern California showed that less than 20% of corneal ulcers were treated in accordance with textbook recommendations (McDonnell, 1992). Performing culture and sensitivity test may be the gold standard but the response of fungi to antimycotic agents is not as good as the response of bacterial infections to antibiotics. The available antifungal agents are limited in their efficacy because of the limited penetration into the cornea, the fungistatic nature, and the development of drug resistance (Carrasco and Genesoni, 2011). Some organisms that were thought to be commensals are proving to be the causes of many unsuspected diseases. The behavior of the microbes has undergone many variations. The two most probable causes are the changes in the organism and its ability to mutate. Many organisms have developed newer protective mechanisms against known antimicrobials. The second cause is the changed immunity in the hosts.

Another important issue regarding the corneal ulcer is that the patients commonly seek help from a wrong source and reach to the hospital only when self treatment or the treatment by unqualified persons fail resulting in the much worse condition. Besides, the elderly people seek help from the adults and younger ones to reach the hospitals and they reach to hospitals when their children are free from their work. In such a situation, the ulcers might have already progressed to worse condition. There is

lack of awareness even amongst the trained community health workers with respect to the adverse effects of the steroids (Sharma, 2011). Lower level and middle level health workers are the trained manpower mainly responsible for primary health care in the rural areas. They are not trained well enough to treat ocular diseases. Lack of appropriate knowledge and skill among health workers and lack of facilities for treating eye diseases are the main reasons why so many patients were found with such extensive lesions. The importance of health education to the general population mainly to farmers should be emphasized.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Filamentous forms of fungi were found in higher number than non- filamentous forms. Among the filamentous fungi, *Aspergillus* was the most frequently isolated followed by *Fusarium* and *Curvularia* respectively. Males were more infected than females. Age group of 31-40 was the most affected.

6.2 Recomendations

1. The safety glasswares or goggles should be worn while working in the fields.
2. Irrational use of corticosteroids should be controlled or avoided.
3. Antifungal drug sensitivity tests need to be developed and standardized to select appropriate antifungal agents.

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APPENDIX A

Clinical and microbiological profile of patients

Name of the patients ……..
Code Number ……..
Date ……..
Age ……..
Sex ……..
Address …….
Occupation …….
Type of cases ……
Duration of symptoms ……
Any ocular trauma a) Yes b) No
Past history of ocular surgery a) Yes b) No
Specimen ……
KOH preparation or LPCB preparation
Gram's stain
Giemsa stain
Fungus culture
Culture on SDA, BA, CA and Thioglycolate broth
Identified organisms ……..
Remarks …….

APPENDIX B

A. Materials requirements

1) Sample: Corneal Scrapings

2) For sample collection and processing:

Glass slides
Petri plates
Kimura scaptula
Glass rod
Topical anesthetic agent (5% Xylocaine)
Conical flasks
Cover slips
Autoclave
Bunsen burner
Incubator
Microscope
Hot air oven
Cotton swab
Beakers
Forcep
Test tubes
Refrigerator
Tray
Cellophane tape
Innoculating loop

Culture media (Hi-media)

Sabouraud Dextrose Agar

Blood Agar

Chocolate Agar

Thioglycolate Broth

Staining and Biochemical reagents

Gram's stain reagents

- Crystal violet
- Gram's Iodine
- Acid alcohol
- Safranine
- Distilled water

10% KOH

Lactophenol cotton blue

Giemsa stain

- 3% methanol
- Giemsa reagent

Human serum

B. Composition and preparation of reagent used

The following reagents were used for the study

1. Potassium hydroxide solution

To make 10% KOH solution

Composition

Potassium hydroxide (KOH)	10gm
Distilled water	100ml

About 10gm of potassium hydroxide pellets were transferred to a clean bottle and 100ml of distilled water was added and mixed until the chemical is fully dissolved. The bottle was labeled and store at room temperature.

2. Lactophenol cotton blue solution

Composition

Phenol	10gm
Cotton blue (aniline blue)	0.04gm
Lactic acid	10ml
Glycerol	20ml
Distilled water	10ml

Cotton blue was weighed and dissolved in water. Warming the water helped to dissolve more quickly. Similarly phenol was weighed in another beaker and stain solution was added. The phenol was dissolved by stirring. Then the lactic and glycerol was added and was mixed well. The bottle was labeled and was stored in a cool dark place.

3. Gram's stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water to make 1 litre	

In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally the volume was made 1 litre by adding D/W.

(b) Gram's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled water	1000 ml

To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled water	25 ml

To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

4. Giemsa stain

Composition

Giemsa powder	3.8gm
Glycerol (glycerine)	250ml
Methanol (methyl alcohol)	250ml

First of all, Giemsa was weighed, transferred to the dry brown bottle which contained a few dry glass beads. Then, 250 ml of methanol was added and was mixed well. Similarly, measured glycerol was added to the stain and mixed well. The bottle was kept in a waterbath at 50-60ºC to help the stain to dissolve and was mixed well at intervals. Then a small amount of the stain was filtered into a stain dispensing container for use.

APPENDIX C

Composition and preparation of media used

The following media were used for the study

Sabouraud Dextrose Agar (Hi- Media)

Composition:

Ingredient	**grams/litres**
Dextrose	40
Peptone	10
Agar	20

Final pH (at 25ºC) 5.6 ± 0.2

About 65gms of Sabouraud Dextrose Agar powder was suspended in 1000ml distilled water. It was then heated to boiling to dissolve the medium completely. Then it was sterilized by autoclaving at 15lbs pressure (121ºC) for 15 minutes and aseptically dispensed 20-25ml into sterile petriplates. Finally the medium was stored in a refrigerator.

Blood Agar Base (Hi-Media)

Composition

Ingredients	**grams/litres**
Beef heart infusion	500
Tryptose	10
Sodium Chloride	5
Agar	15

Final pH (at 25ºC) 7.3 ± 0.2

About 40 gms of Blood Agar Base powder was suspended in 1000ml distilled water. It was then heated to boiling to dissolve the medium completely. Then it was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. It was cooled to 50ºC and 5% v/v sterile defibrinated blood was aseptically added. It was then mixed well and aseptically dispensed 20-25ml into sterile petriplates. Finally the medium was stored in a refrigerator.

Chocolate Agar

About 40 gms of Blood Agar Base powder was suspended in 1000ml distilled water. It was then heated to boiling to dissolve the medium completely. Then it was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. It was cooled to 50ºC and 5% v/v sterile defibrinated blood was aseptically added. It was then mixed well and aseptically dispensed 20-25ml into sterile petriplates. Finally the medium was stored in a refrigerator.

Fluid Thioglycollate Medium (Merck Specialities)

Composition

Ingredients	**grams/litres**
Casein enzymic hydrolysate	15
Yeast extract	5
D (+) glucose anhydrous	5.5
Sodium chloride	2.5
L-cystine	0.5
Sodium thioglycollate	0.5
Sodium resazurin	0.001
Agar Agar	0.75

Final pH 7.1 ± 0.2 at 25ºC

About 29.75 gms of Thioglycollate powder was suspended in 1000ml of distilled water. The powder was completely dissolved by heating in a water bath. Then 15ml of the media was dispensed into the sterile small culture bottles. The bottles were then sterilized by autoclaving at 121ºC and 15 lbs pressure for 15 minutes. Finally the broth media were placed in a refrigerator.

APPENDIX D

Procedures for the different staining methods

10% KOH wet mount preparation

1. A drop of 10% KOH was added in a slide where the smear was prepared.
2. The sample mixed in KOH was covered with a glass cover slip avoiding air bubble and was left for few minutes.
3. Then the slide was observed under microscope at 10X and 40X objectives respectively.

Lactophenol Cotton Blue stain

1. A drop of Lactophenol Cotton Blue was added in a slide where the smear was prepared.
2. The sample mixed in Lactophenol Cotton Blue was covered with a glass cover slip avoiding air bubbles.
3. Then the slide was observed under microscope at 10X and 40X objectives respectively.

Gram's stain

1. The prepared smear was air dried and was heat fixed.
2. The fixed smear was covered with crystal violet stain for 60 seconds.
3. The stain was washed with distilled water and the smear was covered with Gram's iodine for 60 seconds.
4. The smear was decolourised with acid alcohol for 10 seconds and was washed with distilled water immediately.
5. The smear was flooded with safranin for 1 minute and was washed with distilled water.
6. The smear was air dried and was observed microscopically at 10X, 40X and under oil immersion at 100X respectively.

Giemsa stain

1. The dried smear was fixed by covering it with methanol for 5 minutes and the smear was allowed to air dried.
2. The smear was flooded with Giemsa reagent and was left for 30 minutes.
3. The smear was washed with distilled water and was air dried.

4. Then the smear was observed microscopically at 10X, 40X and under oil immersion at 100X objectives respectively.

APPENDIX E

Agewise and genderwise distribution of the patients under study

Age group(Years)	**Male (%)**	**Female (%)**	**Total (%)**
below 10	3 (1.00)	3 (1.00)	6 (2.00)
11-20	13 (4.33)	13 (4.33)	26 (8.66)
21-30	22 (7.33)	23 (7.67)	45 (15.00)
31-40	31 (10.33)	33 (11.00)	64 (21.33)
41-50	31 (10.33)	29 (9.67)	60 (20.00)
51-60	25 (8.33)	18 (6.00)	43 (14.33)
61-70	20 (6.67)	18 (6.00)	38 (12.67)
71-80	8 (2.67)	4 (1.33)	12 (4.00)
81-90	5 (1.67)	1 (0.33)	6 (2.00)
Total	**158 (52.7%)**	**142 (47.3%)**	**300**