

**MICROBIOLOGICAL PROCEDURES FOR DIAGNOSIS
OF OCULAR INFECTIONS**

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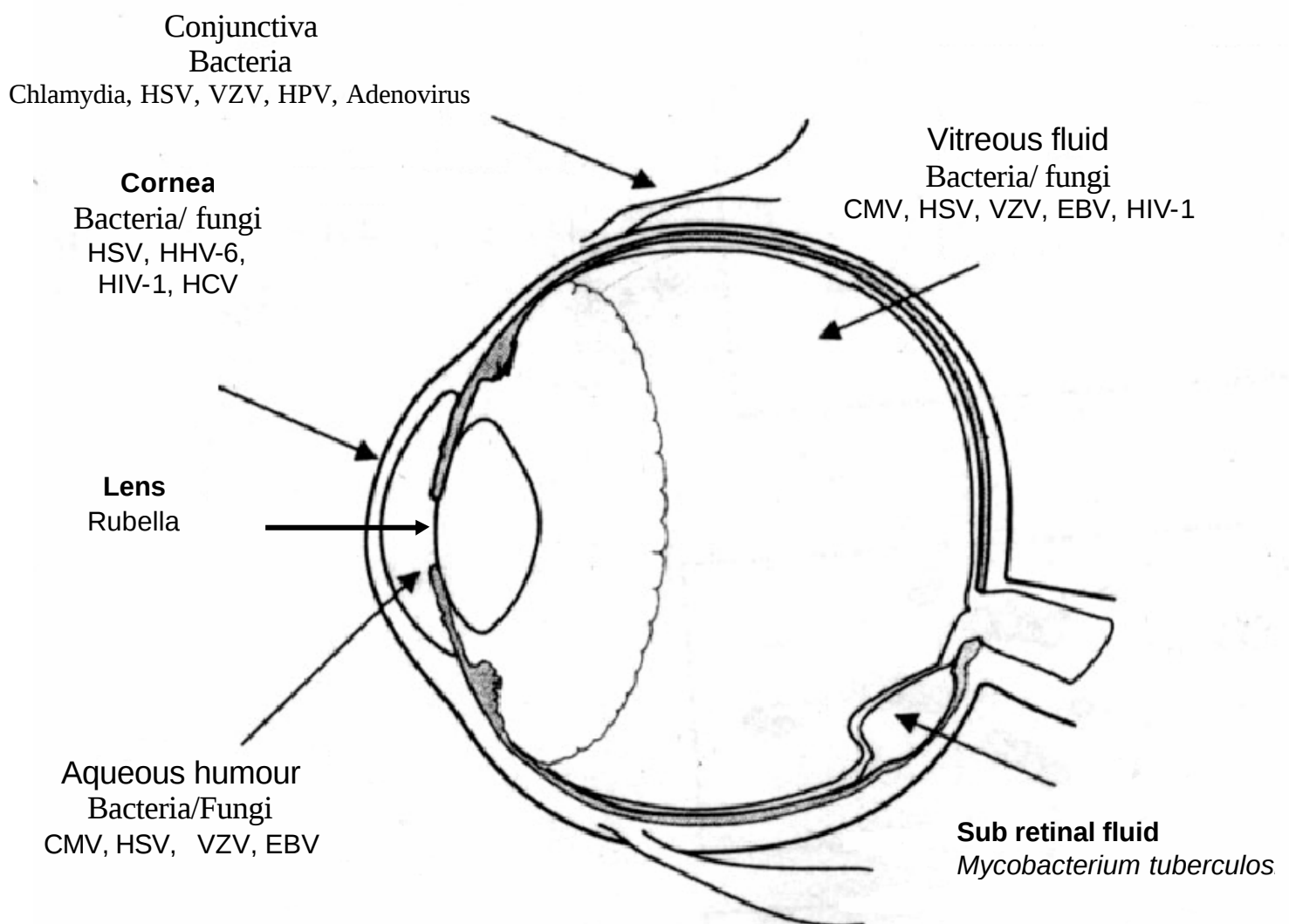
INTRODUCTION TO OCULAR MICROBIOLOGY

Ocular microbiology remains an applied science. The advancements in molecular biology and the newer technologies pave way for better understanding of ocular diseases. Advances in the field of infectious diseases are rapid. The developments have made major contributions in the control and probably even eradication of many types of eye infections. Blinding diseases such as trachoma have been brought under control because of development of rapid diagnostic methods and public health measures. There is dramatic decrease in classical infections of the eye, but new and emerging eye infections are on the rise.

Many opportunistic pathogenic agents are increasingly encountered in ocular infections due to widespread use of topical and systemic immunosuppressive agents, increasing numbers of patients with human immunodeficiency virus (HIV) infection and with organ transplants who are on immunosuppressive therapy. These opportunistic pathogens also cause ocular infections due to increased use of contact lens. The dreaded infections endophthalmitis following cataract extraction and lens implantation often are caused by opportunistic pathogens.

To understand ocular microbiology and ocular diagnostic microbiology, it is essential to have the basic knowledge of anatomy of the eye (Figure 1) and the common microbial agents associated with the ocular infections. The principles involved in mechanism of the ocular surface and parameters intraocular immune mechanisms are useful in understanding ocular microbes. Basic knowledge of pathogenesis of ocular infection and structural consequences are essential in understanding ocular microbes.

Transmission of infection in Ophthalmic practice and the methods of prevention are important public health issues. Microbial agents from the environment. As in other organ systems exposed to environment, ocular surface is colonized by microbial agents which are mainly commensals. These residents induce minimal activation of inflammation and immune responses of the host. The exact microbial population of the ocular surface depends on the age of the host and geographical location and the climate.

Figure 1 . **Anatomy of the eye**

External ocular infections are among the leading causes of ocular morbidity and blindness in developing countries. In spite of constant exposure to infective agents

from environment, conjunctiva and cornea are protected by efficient defense mechanisms. Several risk factors as age, sex, immune status and socio-economic background determine the pathogenesis of infective ophthalmic diseases. Advances in microbiological techniques have made it possible not only to understand the pathogenesis of these infections but also develop better diagnostic methods. Despite dramatic decrease in classical ocular infections, newer infective disease are increasingly encountered.

The conjunctival sac is colonized by bacteria at birth remains so throughout life with changes in the flora due to various factors. Microbial flora mainly consist of *Staphylococcus epidermidis*, *S. aureus*, *Corynebacterium sps.* and *Propionibacterium acnes* and with increasing age, Gram negative bacteria also become part of the flora. Microbial adherence to epithelial surface occurs due to molecular interactions between bacterial surface proteins and protein receptors (integrins) on the cell surfaces. Pili or fimbriae of gram negative bacteria such as *Pseudomonas aeruginosa* play an important role for adhesion to the cell surface.

Bacteria colonizing conjunctival sac produce bacteriocins and inhibitory products such as lactic and acetic acids when help them the necessary competitive advantage to survive and prevent establishment of pathogenic micro-organism. Prolonged usage of topical antibiotics may result in change of microbial flora with implantation of fungal and antibiotic resistant bacteria.

Tear film contains several proteins, electrolytes, amino acids and metabolic products which have anti microbial effects. Lysozyme forming nearly 30% of total tear proteins is active against Gram positive bacteria and accelerates lysis of Gram negative bacteria in the presence of antibody and complement. Other important antimicrobial proteins are lactoferrin, beta lysine, complement and immunoglobulins particularly IgA. Substantial proportions of cellular fractions are made of lymphocytes (700/ml) and polymorphonuclear leucocytes (50/ml) which contribute to removal of particulate materials including micro-organisms. Use of extended wear contact lens produces significant changes in conjunctival microbial flora often with increase in potentially pathogenic micro-organisms. Contact lens disinfectants can select out the microbial agents resistant to them.

NORMAL FLORA OF THE CONJUNCTIVA :

- * Microbial flora is present in the conjunctival sac from birth and is present throughout the life. A very small percentage of population have sterile conjunctival sac.
- * *Staphylococcus* species. and *Diphtheroids* are predominant organisms.
- * Anaerobic bacteria are often present : 0.33%. About 3 -15% population have fungal flora.
- * New born have *Escheicia coli* and *Staphylococcus* commonly and as the age advances are replaced by *Staphylococcus* and *diphtheroids*. Gram negative bacilli are found more often in hospitalised patients; in particular *Pseudomonas sp.*, *Proteus sp.*, *Alcaligenes faecalis* and non-fermenting gram negative bacilli.

MICROBIAL FLORA OF NORMAL EYE :

* AEROBIC : Gram Positive Cocci

<i>Staphylococcus epidermidis</i>	30-80%
<i>Staphylococcus aureus</i>	3-25%
<i>Micrococcus sp.</i>	1-28%
<i>Streptococcus pyogenes</i>	0-03%
<i>Streptococcus pneumoniae</i>	0-03%
<i>Streptococcus viridans</i>	0-01%

Gram Negative Cocci

<i>Moraxella catarrhalis</i>	2-05%
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Gram Positive Bacilli

<i>Corynebacterium species</i> .	5-83%
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Gram Negative Bacilli

<i>Haemophilus influenzae</i>	0-01%
<i>Klebsiella sp.</i>	0-0.5%
<i>Escherichia coli</i>	0-01%
<i>Pseudomonas aeruginosa</i>	0-02%
<i>Moraxella sp.</i>	0-02%

* ANAEROBIC :

<i>Propionibacterium sp.</i>	0-33%
<i>Peptostreptococcus</i>	0-02%
<i>Bacteroides sp.</i>	0-01%
<i>Lactobacillus sp.</i>	0-02%
<i>Clostridium sp.</i>	0-02%

* FUNGUS :

These are transient and are those found in the environment.

* VIRUS :

These are not normal residents.

EXTERNAL OCULAR INFECTIONS

INFECTIONS OF THE EYELID / EYELID GLANDS :

•Blepharitis :

Inflammation of the eyelid margin / Infections of the glands of the eyelid

•Hordeolum :

Acute infections of the glands of Zeis (sebaceous gland) characterised by redness , pain , and swelling of the eyelid

•Meibomitis/ Diffuse Meibomitis :

Multiple infections of the gland and more than one gland is affected

•**Chalazion**

It is a persistent inflammatory response due to ocular bacterial and fungal infections bacterial and cellular elements along with irritative fatty acids in swelling and induration - cosmetically disfiguring. *Staphylococcus aureus* is the most common causative bacterial agent of chalazion.

•**Folliculitis**

Infection of the eyelash follicle *Staphylococcus aureus* is the most common causative bacterial agent

Parinaud's oculoglandular conjunctivitis - *Rochalimaea henselae*

Cannaliculitis - inflammation of the canaliculi

Bacterial infections:

Actinomyces species, Nocardia species, non fastidious anaerobic bacilli
Staphylococci, Streptococci, rarely by *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Lymphoma granuloma venereum* (LGV).

Blepharitis :

Staphylococcal blepharitis : This is a chronic inflammation of eyelid margins colonized by *Staphylococcus aureus* and *Staphylococcus epidermidis*.

It has been shown that the majority of *S. aureus* isolates from blepharitis produce : alpha, beta and delta lysins. Cell-mediated immune response with hypersensitivity to *S. aureus* has been implicated in the pathogenesis of this disease. Baird Parker scheme of biochemical tests for identification of *Staphylococcus* differentiate them from micrococci. For laboratory diagnosis lid margin swabs are collected in case of blepharitis. Sterile cotton tipped swab or calcium alginate swab moistened in Hank's balanced salt solution (HBSS) or brain heart infusion broth (BHIB) or normal saline is rubbed over the lid margin.

Swab is inoculated directly onto Blood agar (BA), Chocolate agar (CA), MacConkey agar (MA) and Sabouraud dextrose agar (SDA).

If pus is present, swabs are used for its collection and BA, CA, MA and BHIB are directly inoculated and at least three smears are made using fresh sterile swab each time.

Phthyrus palperum,

Is not an uncommon eyelash infestation caused by the crab louse *Phthyrus pubis* and its ova, with a clinical manifestation of itching and irritation of lid margin, is diagnosed by identification of the parasite microscopically. It should be differentiated from *Pediculus corporis* and *Pediculus capitis* which are larger.

Bacterial Conjunctivitis :

Even though benign and often self limiting, bacterial conjunctivitis if not diagnosed and treated properly can lead to ocular morbidity of devastating consequences. Being a very common disease, its overall incidence cannot be known as bacteriological investigations are not done routinely.

Types of Bacterial Conjunctivitis :

Hyperacute - *Neisseria gonorrhoeae* *Neisseria meningitidis*

Acute - pathogenic bacteria

Chronic - *Staphylococcus aureus* and *Moraxella lacunata* *Streptococcus pneumoniae* *Haemophilus influenza* *Haemophilus aegyptius*

Since different types of bacteria are normally found in healthy conjunctival sac, it may often be difficult to pinpoint a specific bacterium or a group of bacteria as the causative agents when cultured. Several pathogenic bacteria can cause conjunctivitis but the most frequently associated bacteria are *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (*Haemophilus aegyptius*-Koch-Week bacillus). *Haemophilus influenzae* causes acute purulent conjunctivitis of a longer duration than other organisms and in immunocompromised persons complication of corneal scarring and relapsing conjunctivitis needing systemic antibiotic therapy may also occur.

Conjunctivitis due to anaerobic bacteria mainly due to Gram positive cocci have been reported. Hyperacute purulent conjunctivitis caused by *Neisseria gonorrhoeae* occurs in neonates (Ophthalmia neonatorum) and young adults with complication of ulcerative keratitis resulting in permanent visual loss and corneal perforation,

Streptococcus pyogenes, *Neisseria gonorrhoeae* *Haemophilus influenzae* and rarely *S. Staphylococcus aureus* and *Streptococcus pneumoniae* cause the severe form of pseudomembranous conjunctivitis which should be differentiated from membranous conjunctivitis caused by *Corynebacterium diphtheriae*.

Diphtheritic conjunctivitis is associated with necrosis and sloughing of conjunctival epithelium due to diffusible toxins produced by the bacterium.

Chronic Conjunctivitis : Conjunctivitis caused lasting more than 4 weeks is referred to as chronic conjunctivitis and is usually caused by *Staphylococcus aureus* and *Moraxella lacunata*. The latter organism is associated with angular blepharoconjunctivitis and may cause epidemics.

LABORATORY DIAGNOSIS OF CONJUNCTIVITIS{

Laboratory procedure in ocular microbiology is unique because of small amount of material available for investigation.

- Often number of organisms are small.

- In particular, patients with external eye infection often are on topical antibiotics when referred to for investigation.
- Specimen may contain non-viable micro-organisms and liquid media should be used.
- Fastidious organisms are frequently encountered, therefore enriched media are necessary. With the above factors influencing the outcome of microbiological investigations, inoculation of media are done at 'bedside' and use of transport or preservative media is avoided particularly for bacterial isolation.
- Procedures for viral infections are highly sophisticated and often require prior discussion with laboratory personnel for finalizing the acceptable methods for collection of specimens for isolation. It is complicated by the presence of microbial flora in the conjunctival sac from birth and throughout the life and therefore a semi-quantitative and quantitative methods are necessary for bacteriological investigations of conjunctival specimens.

REQUIREMENTS FOR COLLECTION OF EXTERNAL OCULAR SPECIMENS

Collection is preferably done by Ophthalmic surgeon.

Conjunctival material may be collected by the trained technologist from Microbiology laboratory. .

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A collection kit must readily be available and it includes :

1. Spirit Lamp	1 No.
2. Match Box	1 No.
3. Kimura Spatulas	2 No.
4. Disposable sterile blades	10 Nos.
5. Spirit in coplin jar	1 No.
6. Clean microscopic slides (Preferably new ones)	1 Box.
7. Diamond marking pencil	1 No.
8. Glass marking pencil/pen	2 Nos.
9. Topical anaesthetic	1 Vial with droppers
10 Fresh Growth media solid	As required
11 Transport liquid (2 SP/HBSS)	4 Nos.
12 Sterile cotton tipped swabs	6 Nos.
13 Coplin jar containing 95% Methyl alcohol	1 Jar
14 Coplin jar with cold acetone	1Jar
15 10% Potassium hydroxide dropper	1 No

COLLECTION OF SPECIMENS*

Lid Margin :

- No topical anaesthetic is needed.
- Sterile cotton tipped swab or calcium alginate swab moistened in HBSS/ brainheartinfusion broth (BHIB) is rubbed over the lid margin.
- Swab is inoculated directly.

Blood agar (BA) Preferably a single petri dish .
 Chocolate agar(CA) medium is used for each specimen
 .Brucella blood agar (BBA) from each eyelid. .

If pus is present, swabs are used for its collection and BA, CA, MA, BBA and BHIB are directly inoculated and atleast three smears are made using fresh sterile swab.

Collection of Conjunctival material (Conjunctival Swab)



- Sterile moistened cotton swab or calcium alginate swab are used. Bacterial culture medium such as BHIB or normal saline may be used for moistening the swab.
- Patient is requested to look up, the lower eye lid is pulled down using thumb with an absorbing tissue paper and moistened swab is rubbed over the lower conjunctival sac from medial to lateral side and back again. The procedure is often slightly painful.
- Sterile plastic (soft) bacteriological loop may be used for collection of material.
- Avoid collection of tears only. Swab is directly inoculated onto blood agar (aerobic incubation) chocolate agar (5-10% CO₂) and BBA (anaerobic). On solid media main inoculum only is made and further streaking is done in the laboratory.

Collection of Conjunctival Scraping :

This method is particularly used for detection and isolation of *Chlamydia*

trachomatis and viruses. It is used in most laboratories reveals just the presence of the bacteria which may only be the normal resident whose role in pathogenicity can not be evaluated.

Topical anaesthetic is placed over the eye and wait until the anaesthetic effect is developed. - 2-3 minutes for effective application of topical anaesthesia,

- Meanwhile the Kimura spatula is dipped into spirit and is burnt by passing it over the spirit lamp. (Take care not to bring the flame near the coplin jars containing spirit, methyl alcohol and acetone, which are all inflammable).
- Allow the spatula to cool before giving them to the surgeon (about 2 minutes).
- The scrapings are taken, from the everted eyelid and the material on the spatula is inoculated into BA, CA, and BBA, directly.
- Material taken by the spatula second time is used to making smears - 6 Nos.
- Scrapings should be taken from the representative part of conjunctiva in both upper and lower eyelids.

Note : * Kimura spatula is sterilized by flaming and cooling or wiped with 70% Ethyl alcohol and allowed to dry.

- The sterile surgical blade (disposable) also may be used; it is advisable to use the blunt edge of the blade for scraping; bleeding should be avoided.
- Material taken by the spatula second time is used to making 6 smears which are needed for direct diagnosis by use of different staining methods.
- Scrapings should be taken from the representative part of conjunctiva in both upper and lower eyelids.
- The sterile blade (disposable) also may be used; it is advisable to use the blunt edge of the blade for scraping; bleeding should be avoided.
- The scraping should be inoculated into
 1. Sucrose phosphate broth (2 SP for Chlamydia)
 2. HBSS (for viruses) in 1 ml amounts for transport to the laboratory for culture.

It may be noted that 2 SP can also be used for transport of specimens for isolation of viruses. Further procedures for detection of *C. trachomatis* and viruses are discussed under the respective headings.

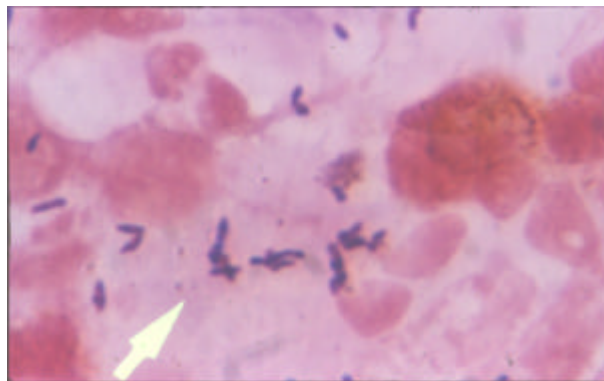
Three to four smears are taken on clean glass slides within an area defined with a wax pencil on the reverse.

The smears are stained as follows;

1. Gram Stain

- 2 Geimsa Stain
- 3 Special stains - Ziehl Neelsen Acid Fast Stain or Immunoflorescent staining for chlamydiae /Adeno viruses (the smears taken for immunoflorescent staining are immediately fixed either in cold acetone or in 95%alcohol).

Gram stained smear of conjunctival swab showing the pus cells (polymorpho leucocytes) and typical coryneform diptheriodes in clusters and in singles and pairs



QUALITATIVE CULTURE FOR BACTERIA FOR COJUNCTIVAL SECRETIONS - SEMI - QUANTITATIVE ROCEDURE

The sterile swab is wetted with HBSS. Collect conjunctival material rubbing the swab over the lower conjunctival fornix from medial to the lateral side.

The swab is immersed in 1 ml of Hank's Balanced Salt Solution (HBSS) and excess applicator stick is broken and the tube is screw capped

- The tube is vortexed for 60 seconds.
- The swab is pressed on the side of the tube to express all conjunctival material and discarded.
- 100 μ L of HBSS is inoculated each on to Blood agar and Chocolate agar and is spread on the surface of the media.
- A streak of *Staphylococcus aureus* is made across the inoculum of BA for isolation of *Haemophilus*.
- Blood agar is incubated at 37°C aerobically and Chocolate agar is incubated in 10% CO₂ atmosphere at 37°C overnight.

- Enumerate colonies and report as scanty, moderate, heavy and very heavy growth according to the format given below.
- Identify the organisms as per the standard methods and do antibiotic sensitivity and report.
- Carry out catalase and oxidase tests with colonies.
- Make smears and do gram stain.
- * Record colony characteristics including haemolytic property.
- * Do biochemical tests as listed in the laboratory.
- * Identify the bacterium to the nearest Genus and species.
- * Categorize the organism as "Culture positive" or "Culture negative" according to the number of viable organisms and threshold criteria enclosed.

Threshold Criteria for Judging "Culture Positive" Specimens

Note : An ocular specimen is considered "Culture Positive" if colony count equals or exceeds the threshold values given below for any of the following groups of organisms.

Group I: Threshold=1CFU/100mL(i.e.any counts)1-10 (scanty)

Streptococcus, Group A, Beta hemolytic (S. pyogenes)
Streptococcus pneumoniae
Staphylococcus aureus
Citrobacter sps
Enterobacter sps
Escherichia coli
Klebsiella sps
Proteus/Morganella
Serratia marcescens
 Other Enterobacteriaceae
Neisseria gonorrhoeae
 Other Moraxella sps
 Other Neisseria sps
Acinetobacter sps
Achromobacter sps
Haemophilus sps
Pseudomonas aeruginosa
 Other Pseudomonas sps

Group II : Threshold = 10 CFU/100 mL (Moderate) 10-50

Streptococcus Group B (Beta or non -hemolytic)
Streptococcus Group C (alpha, beta or non- hemolytic)
 Other Streptococcus (Group D, G; nongrouped; viridans)

Moraxella (Branhamella) catarrhalis.

Group III : Threshold = 100 CFU/100 mL (Heavy) 50-100

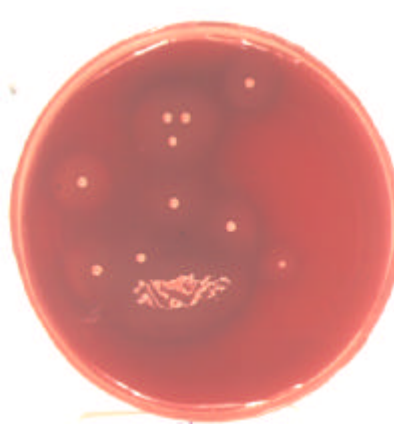
Staphylococcus epidermidis
Other coagulase negative *Staphylococcus*
Micrococcus sps / *Bacillus* sps

Group IV :

Threshold = 100 and above CFU/100 µl – *Corynebacterium* sps
(diphtheroids).

In our experience the above threshold values have correlated well with the clinical picture and therapeutic outcome of primary bacterial conjunctivitis.

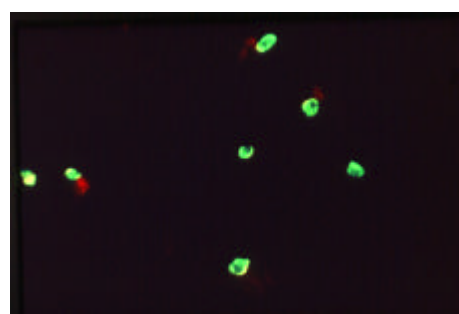
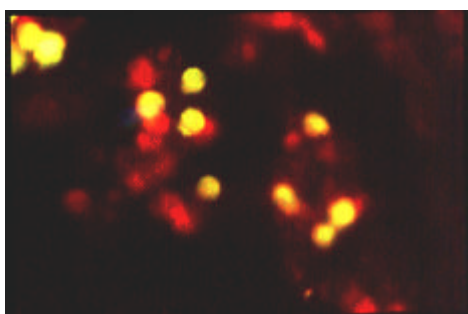
Growth of *Propionibacterium acnes* colonies on Brucella Blood agar



Chlamydia Conjunctivitis :

Chlamydia trachomatis, an exclusively human pathogen, is a non-motile, Gram negative intracellular bacterium having a unique developmental cycle in the infected cell. Metabolically inactive elementary body (EB) which is infectious enters the host cell (columnar epithelial cell) and develops into a reticulate body (RB) which divides rapidly by binary fission leading to a release of infectious

Immunofluorescence staining showing the reticulate bodies of *Chlamydia trachomatis* grown on McCoy cell line culture.



EBs to infect fresh adjacent cells, EB possesses heparin sulphate like molecules on their surfaces which help them to adhere to host cells which endocytose the organisms within the cytoplasm, *C. trachomatis* multiplies to form an inclusion. The organism possesses a very small chromosome with only very few protein coding capacity. Therefore, they depend on the host cell for many nutrients and obtain their energy requirement from host derived ATP.

Since the organism cannot synthesize folic acid, it is susceptible to sulphonamides. Glycogen is produced in large quantities surrounding the multiplying Chlamydia forming a matrix in the cytoplasm and can be visualized as brown staining inclusion by iodine staining. Persistence of chlamydial infection results in recurrences and is due to several factors such as deprivation of essential amino acids, continued presence of antibiotics topically applied into conjunctival sac and inhibition of chlamydial replication due to interferon gamma. Infected epithelial cells show, on Giemsa staining, distinct inclusions which stain deep blue almost capping the nucleus and are called Halberstaedter Prowazek (HP) bodies. *C. trachomatis* is the aetiological agent of trachoma, inclusion conjunctivitis and lymphogranuloma venereum.

Classical trachoma :

It is potentially blinding disease caused by serovars A, B, Ba and C and spreads from eye to eye through flies and direct contact with infected materials. This disease is the largest, single cause of preventable blindness worldwide. Inclusion conjunctivitis sometimes indistinguishable from trachoma is caused by serovars D, E, F, G, H, I, J and K which are found in the genital tract and are sexually transmitted. Often referred to as an adult chlamydial conjunctivitis. Positive conjunctival immunofluorescent staining for *C. trachomatis* in patients of non-gonococcal urethritis has been demonstrated indicating latent carrier state of the organisms in conjunctiva.

Chlamydial infection have been reported in more than 25% of newborn conjunctivitis has a range of clinical features indistinguishable from viral, bacterial and allergic

conjunctivitis or keratoconjunctivitis and clinical presentation may be acute or chronic. **The definitive diagnosis is achieved only by isolation of the organism.**

Several diagnostic methods are available to detect *C. trachomatis* in direct smears from the lesions such as Giemsa stain cytology, fluorescent antibody test (FAT), enzyme immune assay (EIA) using either monoclonal or polyclonal antibodies.

The shell vial assay for detection on MOMP antigen of *C. trachomatis* amplified in cyclohexamide treated McCoy cell cultures is the recommended procedure for isolation of the organism. The amplified antigen is detected either by Giemsa or iodine staining but preferably by specific immunoperoxidase or fluorescent antibody test.

The drug of choice in the treatment is tetracycline used both topically and systemically. Macrolides are also useful. In vitro studies on laboratory maintained strains of *C. trachomatis* have shown that β -lactum drugs have no useful activity but fluorinated quinolones have inhibitory action. The first ever study on drug susceptibility of the largest number (27 strains) of clinical isolates of *C. trachomatis* at Sankara Nethralaya, Chennai showed a high in vitro resistance to tetracycline and a relatively high resistance to ciprofloxacin.

Viral Conjunctivitis :

Viral conjunctivitis, a highly infectious condition, is a self limiting disease with low morbidity. An acute follicular conjunctivitis of sudden onset often with respiratory and systemic symptoms and with involvement of the other eye within a week may clinically be diagnosed as viral conjunctivitis. Preauricular lymphadenopathy, lid oedema, conjunctival haemorrhages and corneal changes are often associated with this condition. Adenoviruses are the most frequent causes of viral conjunctivitis occurring in sporadic and epidemic forms. Mild, nonspecific follicular conjunctivitis is the most common form of clinical presentation and is caused by adenovirus serotypes 1,2,4 and 6.

Epidemic keratoconjunctivitis (EKC)

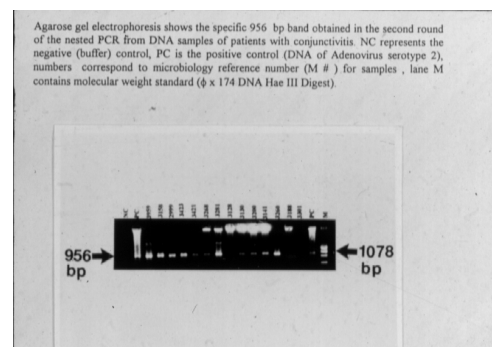
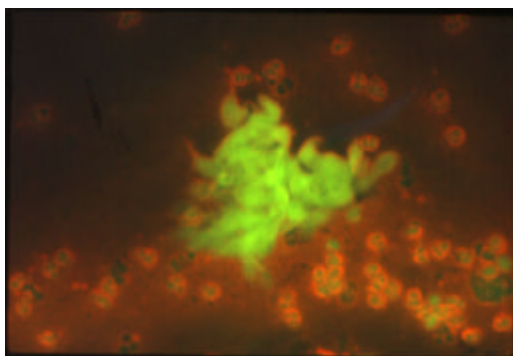
It has an incubation period of 8-10 days and occurs as sporadic or in clusters and epidemics and most often is associated with serotypes 8, 19 and 37 and less often with serotypes 3, 4, 7, 10, 11 and 21.

Pharyngo conjunctival fever (PCF)

It is characterized by fever, pharyngitis, conjunctivitis and other systemic symptoms and signs usually seen in school going children and young adults occurs in outbreaks. Sporadic cases are seen in all age groups. PCF has an incubation period of 6-9 days. Serotypes 3, 4 and 7 are commonly associated and less common types are 1, 11, 14, 16-19 and 37.

These viruses also cause non-specific conjunctivitis and chronic papillary conjunctivitis. The viruses spread rapidly in the community as a result of respiratory tract to eye, eye to eye and via infected tissues and clothes and other fomites and contaminated swimming pools. Laboratory diagnosis of adenoviral conjunctivitis consists of detection of virus by direct methods and isolation of the infecting agent, the most sensitive method and isolation of the infecting agent, the most sensitive method of diagnosis of adenovirus conjunctivitis is its isolation by conventional test tube cell cultures.

Immunofluorescence staining showing the infected cells positive for Adenovirus in the direct smear of conjunctival swab



Enterovirus 70 and Coxsackie A24 can cause bilateral follicular conjunctivitis of sudden onset with conjunctival haemorrhages, transient keratitis. Enterovirus 70 caused a pandemic of acute haemorrhagic conjunctivitis during the years 1969-71. Neurological complications have been noted. During 1970, a large epidemic of acute haemorrhagic conjunctivitis due to Coxsackie A24 involving Asia and Africa occurred. Primary infection with herpes simplex virus (HSV) may occur in newborn (ophthalmia neonatorum) children and young adults.

Follicular conjunctivitis:

Eyelid vesicles, preauricular lymphadenopathy and sometimes ulcerative blepharitis, usually unilateral may be diagnosed as due to HSV. Recurrent herpes occurs in about 25% of patients and cornea is more than involved.

Varicella zoster virus (VZV) may cause ocular complications, following reactivation of latent endogenous virus and these occur in nearly 60 to 70% of patients with involvement of ophthalmic division of trigeminal nerve. A papillary, follicular or membranous conjunctivitis may occur. In 65% of cases cornea is affected. Distinctive skin eruptions on the eyelid and forehead are diagnostic. Laboratory diagnosis of HSV and VZV conjunctival infections are similar to what is detailed under infectious keratitis. Acute follicular conjunctivitis may be caused by Influenza A virus, Newcastle disease virus and Cytomegalovirus. Molluscum contagiosum

may cause chronic toxic conjunctivitis. Pearly white umbilicated nodules on histopathological examination confirms the diagnosis.

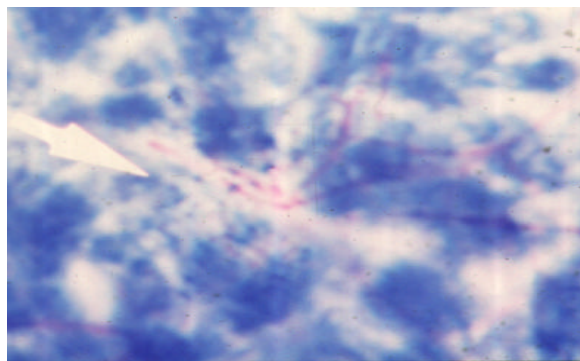
Miscellaneous ocular specimens : Lacrimal system :

Lacrimal apparatus infections

- Dacryoadenitis - inflammation of the lacrimal gland

Most common causes of infection in lacrimal system are *Nocardia*, species *Actinomyces* and anaerobic bacteria, or a mixed flora of both gram positive and gram negative bacteria. Therefore apart from aerobic cultures, anaerobic cultivation is necessary. Pus from Lacrimal sac may be obtained by slight pressure over the lower eyelid at the inner canthus and pus would come out of the punctum from infected canaliculus.

Modified Acid fast staining of pus from canaliculitis showing the acid fast filamentous form of *Nocardia asteroides* in the Canalicular pus



Infections of the sclera

Scleritis :clinical presentation

localised firm tender nodule scleral ulcer /abscess ; relatively uncommon inflammation
pyogenic / granulomatous / immune mediated

Other miscellaneous ocular specimens:

Pus from the Orbit /Infected buckle, suture Iris tissue Eviscerated material
Scleral biopsy Fine Needle Aspiration Biopsy (FNAB) Sub retinal mass

Laboratory procedures :Similar to those used for other ocular specimens.

INFECTIOUS KERATITIS : (MICROBIAL KERATITIS)

Introduction

Infective Keratitis (microbial keratitis) is a major ophthalmic problem often leading to corneal blindness. A wide spectrum of microbial agents produce corneal infections which should be considered a medical emergency. A rapid aetiological diagnosis may help in initiating an aggressive specific treatment for a cure or minimize scar formation.

Clinical features of infectious keratitis may help in the initial diagnosis regarding the etiology. Clinical characteristics of bacterial keratitis caused by individual bacteria are so much overlapping, it is virtually not possible to identify the causative agent. Bacteria by virtue of their toxins, adherence capabilities, invasiveness or strain differences in within a species may produce different types of clinical picture. The variation in the clinical feature may be related to the varying types of contact lenses used or with types of trauma or previous scar in the cornea due to a virus infection. In general Gram-positive bacteria tend to produce discrete, small abscess like lesions whereas Gram-negative bacteria produce diffuse rapidly spreading necrotic lesions.

Patients with infectious corneal ulceration complain of pain, watering, foreign body sensation reduces and reduced vision. Acute pain with watering and rapidly spreading corneal ulcer is likely to be due to *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*. Indolent corneal ulcers may be caused by *Staphylococcus aureus* or *Moraxella* species. Gram-negative corneal ulcers produce marked eyelid edema and conjunctival chemosis. Hypopyon is commonly associated with corneal ulcers of any of the aetiological origin, but is an important sign of Pneumococcal or *Pseudomonas* ulcer. Hemorrhagic hypopyon is associated with pneumococcal or Herpes simplex virus corneal ulcer.

Fungal Keratitis :

Corneal infection of fungal aetiology is very common and may represent 30 – 40 percent of all cases of culture-positive infectious keratitis in South India. Of these, *Aspergillus* and *Fusarium* are responsible for 70 percent of cases. These affect young, immunocompetent healthy adults, more often from rural areas. A history of trauma with organic matter is elicited in a significant percentage of cases. The ulcer commences insidiously and runs an indolent course. It begins at the

midperiphery of healthy cornea in the exposed areas. The epithelium may show defect at the site of infiltration or epithelial defect would have healed with deep stromal infiltrate or endothelial plaque. The ulcer spreads towards the center of the cornea. Moderate hypopyon or cheesy hypopyon is frequently noticed. In rare cases one may see a haemorrhagic hypopyon. The ulcer base has a raised, wet, soft creamy to grayish-white or yellowish-white infiltrate without mucous or exudates. It has feathery or hyphate borders. In the early stages a dendritic pattern may be seen leading to misdiagnosis and treatment with antiviral drugs. In advanced stages with involvement of the whole cornea the typical clinical signs of fungal ulcer become obliterated. Satellite lesions and immune ring, which are infrequent, may assist in diagnosing fungal ulcer. Endothelial plaque and posterior corneal abscess are seen more frequently than described earlier. In pigmented ulcers (chromomycosis) the brown, dark brown or black pigment covering the ulcer base is the unique clinical sign caused by pigmented fungi (dematiaceous). In some of these eyes the slough is dry, tough and leathery and one usually needs a blade to remove it. These ulcers heal very slowly.

Keratitis caused by *Candida* is extremely rare in India and should be differentiated from staphylococcal and *Moraxella* ulcers. Stromal herpes and other low virulent bacterial ulcers should be considered in the differential diagnosis. All these clinical features mentioned earlier may be masked or altered by using native medicines, steroids or / and minor surgical procedures. Although fungal ulcers spread very slowly, corneal perforation can occur within 5 to 6 days from the onset as happens in *Pseudomonas* ulcer.

Acanthamoeba Keratitis :

This parasitic infection is now reported with increasing frequency throughout the world. Trauma with organic matter, exposure to muddy or brackish water are the major predisposing factors. Delayed diagnosis is common. Even though severe pain is considered as the characteristic clinical symptom, there is no marked difference in the symptom between *Acanthamoeba* keratitis and fungal keratitis. A history of unsuccessful treatment by several ophthalmologists with various drugs ophthalmic topical preparations is more often the rule than an exception. *Acanthamoeba* keratitis is usually never suspected or diagnosed during the first visit. Ring of stromal infiltration at midperiphery of the cornea without involving the pupillary area with intact gray or hazy epithelium is noticed in a high percent of cases. Superficial punctate keratitis, small dendrites, subepithelial keratopathy, satellite lesions could be other variants. Radial keratoneuritis is very rare. Usually there is hypopyon but it may be seen in about 20 percent patients. The ulcer remains superficial for several weeks. Typically the disorder evolves over several weeks as a gradually worsening keratitis with periods of temporary remission. A higher index of suspicion is the key to diagnosing *Acanthamoeba* keratitis. Keratitis due to Herpes simplex, atypical *Mycobacteria* and fungi should be thought of in the differential diagnosis.

Viral Keratitis :

Among the causative agents of viral keratitis, Herpes simplex virus (HSV) infection is the most important one as it often leads to blindness. Among the two types of HSV, type I is more commonly associated with this condition. Type II virus keratitis is found in 20 percent of infants born with HSV infection. IN adults the recurrence rates of Herpes simplex keratitis (HSK) are about 25 percent within 1 year and 33 percent within 2 years. Various nonspecific stress factors, e.g., trauma, fever, menstruation, psychological stress, climatic changes are implicated as precipitating factors (Table1). The type of treatment at the beginning of the disease has no apparent effect on recurrence. Recurrent HSV infection manifests in the following varied forms.

Herpes simplex virus infection

Infection HSV	Predominant Virus Type	Outcome	Recurrence
<i>Ocular</i>	1	Vision impairment	Yes
Orofacial	1,2	Resolution	Yes
Genital	2,1	Resolution	Yes
Neonatal	2,1	Retardation	No
Disseminated	1,2	Resolution or death	No
Encephalitis	1	Death	No
Meningoencephalitis	2	Resolution	No

Corneal Epithelial Lesions :

Dendritic Keratitis :

The most frequent manifestation of herpetic ocular infection is the branching linear epithelial ulcer referred to as dendritic keratitis. Superficial herpetic infections of the cornea, appearing as first or recurrent attacks, are almost invariably accompanied by partial or complete loss of corneal sensation is common. Although this sensory loss can usually be detected within a day or two of the onset of the attack, it can be delayed for as long as 11 days. In antibiotic – or placebo-treated patients, most cases of dendritic keratitis last 7 to 14 days, although some may persist for 25 days or longer. Unfortunately, dendritic keratitis may usher in some of the more prolonged forms of the disease. Atypical and resistant forms of dendritic keratitis are seen in immunocompromised and AIDS victims.

Marginal Herpetic Keratitis :

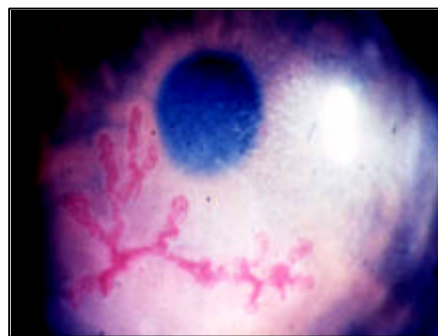
Dendritic and geographic forms near the corneal limbus tend to run a longer course and to respond less readily to antiviral chemotherapy than other herpetic

lesions. The marginal lesions often have underlying corneal infiltration as well as epithelial staining and thus are mistaken for bacterial or other marginal (“catarrhal”) infiltrates. In India, marginal herpetic lesions are more frequently reported after penetrating keratoplasty and cataract surgery.

Geographic or Amoeboid Herpetic Ulcers :

Occasionally a linear dendritic figure progresses to a broad area of epithelial involvement with irregular angulated borders (“geographic” or “amoeboid” ulcer). These lesions have a much longer clinical course, often of many months and often follow the injudicious use of topical corticosteroids for the treatment of dendritic keratitis.

Geographic” or “amoeboid” ulcer stained with Rose bengal stain .



Indolent Keratitis :

Necrotic stromal keratitis may be associated with relatively large epithelial defects or it may evolve from treated geographic ulcers. These incident forms have a particularly prolonged course and usually have profound corneal anaesthesia, do not respond to therapy with topical antiviral drugs, and are not associated with infectious HSV. Along with the loss of epithelium, there is marked swelling of the cornea, accompanied by folds in Descemet’s membrane and marked discomfort. The term “trophic” ulcer is often applied to these indolent ulcers because of the marked loss of sensation.

In patients with severe localized stromal keratitis, necrosis of the deep tissues leads to loss of the overlying epithelium. These round or oval, relatively deep ulcers tend to have straight borders and were referred to by Gunderson as “metaherpetic ulcers”. They are usually accompanied by marked corneal anaesthesia and run a prolonged course.

All these indolent ulcers bear some resemblance to recurrent epithelial erosions of the corneal in which there is a failure of the epithelium to attach to underlying basement membrane. Some of these indolent ulcers are complicated by microperforation.

Herpetic Stromal Keratitis :

Superficial Keratitis with epithelial lesions :

It is not uncommon to see opacification of the anterior corneal stroma directly beneath the site of dendritic lesion. This superficial opacity has the same form as the ulcer and persists long after the epithelial lesion has healed, particularly in cases treated with antiviral agents.

Disciform Keratitis :

This is a central round (Disciform) lesion of the cornea, with opacity and swelling of the corneal stroma. It may follow an epithelial lesion immediately or may appear long after the original epithelial lesions have healed. Disciform keratitis due to HSV is associated with marked anaesthesia and with keratic precipitates immediately beneath the lesion on the corneal endothelium. It may also follow infections with vaccinia, herpes zoster, mumps and varicella but is most frequently associated with HSV ocular infections. It may run a course of a few weeks to several months. The milder cases tend to heal without sequel, but severe cases sometimes progress to permanent stromal scarring.

Diffuse Stromal Keratitis :

In patients with previous ocular herpetic infection, the deep layers of the corneal stroma may be diffusely affected, often without any typical epithelial herpetic lesions. This is most likely to occur after the use of corticosteroids and the lesions may have a prolonged course.

Endothelial Involvement :

Herpes simplex endothelitis is a recently recognized clinical entity. Clinically endothelitis presents with mild stromal oedema, few medium sized keratic precipitates, aqueous flare and cells. The condition is usually associated with secondary glaucoma due to trabeculitis. Herpes simplex virus antigens have been demonstrated in human endothelial cells in patient suffering from endothelitis, disciform keratitis and anterior uveitis. In a case of endothelitis we isolated vesicular virus in the aqueous humor of the patient .

Herpetic Limitis :

This is characterized by the presence of localized inflammation of the deep corneal stroma at the limbus with an adjacent sector involved by scleritis. The corneal lesion often has a wide base at the limbus and narrows towards the centre of the cornea. These lesions may represent sclerokeratitis or may evolve from marginal epithelial herpetic lesion.

Herpes Zoster Ophthalmicus (HZO) :

It occurs due to activation of latent varicella zoster virus infection which persists after primary varicella infection. Corneal complications occur in 40 percent of cases of HZO. The most common findings are dendrites and punctate keratitis. The dendritiform figures are not excavated, but are made of swollen, heaped cells and have a gray plaque-like appearance; unlike the delicate pattern of HSV dendrites, the zoster dendrite is more coarse, rosy and stellate, also the terminal bulbs seen in simple dendrites are absent. They resolve without treatment within one month.

Other findings in cases with corneal involvement in HZO are punctate keratitis and mucous plaques.

LABORATORY METHODS OF DIAGNOSIS OF INFECTIOUS KERATITIS

Corneal inflammation in general is known as keratitis,

Inflammation with a significant loss of epithelium is called corneal ulcer.

There are many different types of corneal ulcers, but they all produce very much the same symptoms –

Pain, blurred vision, photophobia and watering.

A wide spectrum of microbial organisms can produce corneal infections and consequently the therapeutic strategies may be variable.

The types of microorganisms which can invade cornea are bacteria, fungi, viruses, and parasites.

Most of these organisms come from outside, through the epithelium and into the cornea, Microbial keratitis results from a complex interaction between a protean array of pathogens and a diversity of host responses.

The main predisposing risk factors for keratitis :

Trauma, contact lens wear and ocular surface compromise.

Early diagnosis and prompt initiation of appropriate therapy are key factors in successful management of keratitis.

Clinical features are not consistent and a diagnosis should **not** be made on clinical features alone.

The approach to any corneal infiltrate is to regard it as ‘infected until proved otherwise’. This high index of suspicion mandates full microbiologic workup of most corneal ulcers, except where history and examination clearly indicates a non infectious etiology.

The signs of infectious keratitis are cells in the precorneal tear film,

central location of ulcer,

axial extension, purulent discharge and hypopyon,

All the above signs warrant prompt microbiological investigations.

Owing to the considerable overlap in the clinical appearances of corneal ulcers due to various microorganisms, a standard basic laboratory methodology which encompasses the techniques to recognise the different causative agents should be included. Figure 1 depicts a procedural flow chart to isolate the causative organism.

COLLECTION AND PROCESSING OF CLINICAL SAMPLES OF CORNEAL ULCERS OR KERATITIS.

Cornea : It is advisable to culture the ipsilateral conjunctiva in the standard method described above as it is considered that in the event of the corneal ulcer is culture negative, the organisms grown from conjunctiva may be considered as the chlamydia infections but not so for bacterial and fungal diseases.

- Topical anaesthetic is applied.
 - Wait for 3-5 minutes for draining of the anaesthetic.
 - Purulent material is removed by a sterile cotton swab and discarded.
 - Lid speculum may be used
 - (to prevent eyelashes contaminating
 - the Kimura spatula) or assistant should hold the lids apart.
- Corneal scraping should be done with the aid of slit lamp.
- Kimura spatula (sterilised by flaming and colling or wiping with 70% ethyl alcohol and drying) is used to obtain corneal material. Surgical disposable blade may also be used.
- Material is inoculated directly onto Blood agar (aerobic) chocolate agar (CO₂) and Brucella blood agar (anaerobic) media with an 'X' and into BHIB and ThioB or cooked meat medium.
- Smears of about 5-6 are made on clean microscopic slide.
- On one smear (thick enough) 10% KOH is placed and covered with cover slip.
- Broth moistened swab may be used to transfer corneal material from the spatula blade to liquid media.



Note : Routine cultures for anaerobic bacteria are not needed, but the surgeon and laboratory worker should be aware that anaerobic bacteria often the causative agents of the lesion.

COLLECTION OF CORNEAL SCRAPINGS :

- o A drop of local anaesthetic without preservative is instilled , and with the help of the slit lamp or operating microscope ,
- o the edge of the ulcer is firmly scraped using Bard Parker blade No. 15' after removal of debris or discharge in the vicinity.
- o Several scrapings are collected and used in a sequence to inoculate culture media and prepare smears as given in the table. (Table 1).
- o For viral and chlamydial cultures corneal scrapings are collected in Hanks Balanced Salt Solution(HBSS) and sent to the laboratory immediately.
- o In case of delay the specimens are stored at -20⁰C for viral and chlamydial isolations.

COLLECTION OF CORNEAL BIOPSY :

The material for diagnosis should include an adequate area of cornea affected clinically by the inflammatory or ulcerative process. The material collected is processed similar to corneal button as given below.

COLLECTION OF CORNEAL BUTTON :

The corneal button removed by surgery is sent to Microbiology Laboratory in a sterile container immediately. The corneal button and the corneal biopsy tissues are cut into small bits in a small sterile petridish following all aseptic precautions , and inoculated onto various culture media, as given in table 1.

Crushed smears are also made to be used for various staining methods as given in **Table 1**

TABLE 1

SEQUENCE OF CULTURE MEDIA AND SMEARS FOR PROCESSING OF CLINICAL SAMPLES OF SUSPECTED INFECTIOUS KERATITIS SAMPLES.

Culture media used	Incubation period and conditions of growth for Culture Media		
	Growth Condition		
	Temperature (⁰ C)	Atmosphere	Period (Days)

Blood agar	35	Aerobic	10
Brucella blood agar	35	Anaerobic	10
Chocolate agar	35	5-10% CO ₂	10
Sabouraud's dextrose agar	25	Aerobic	15
MacConkey agar			
Brain heart infusion broth	25	Aerobic	10
Thioglycolate broth/Roberson cooked meat medium	35	Aerobic	10

Optional Culture Media :

Lowenstein Jensen medium/Kirchner's liquid medium.	35	Aerobic	21
Non-nutrient agar (two plates) - 1	35	Aerobic	10
- 2	25	Aerobic	10

DIRECT SMEARS :

Six smears are taken on clean glass slides within an area defined with a wax pencil on the reverse.

The smears are stained as follows;

1. Potassium hydroxide
2. Calco flour White
3. Gram Stain
4. Geimsa Stain
5. Special stains - Ziehl Neelsen Acid Fast Stain or Immunoflorescent staining for chlamydiae and viruses (the smears taken for immunoflorescent staining are immediately fixed either in cold acetone or in 95%alcohol).
6. Optional Special stains like Periodic Acid Schiff (PAS) stain or Gomori Methenamine silver (GMS) stain.

PROCESSING AND INTERPRETATION OF DIRECT SMEARS :

A. POTASSIUM HYDROXIDE (KOH) WET PREPARATION :

A10 % solution of potassium hydroxide is used to visualize fungal elements in corneal scrapes, in a homogenous background of corneal tissue digested by KOH

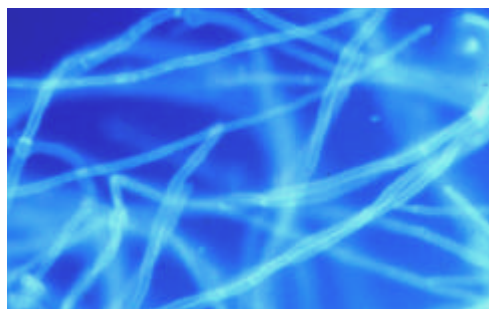


Many branching septate fungal filaments are seen

B. CALCOFLOUR WHITE (CFW) STAIN WET PREPARATION :

A drop of 10% KOH and a drop of CFW is added to the smear, and covered with a coverslip without introducing air bubbles and is observed under florescent microscope using filters V-2A at EX 380-420 BA 450. Fungal filaments and Acanthamoeba appear bright white with CFW stain.

CALCOFLOUR WHITE (CFW) STAIN OF A CORNEAL SCRAPING



Plenty of Septate fungal filaments are seen

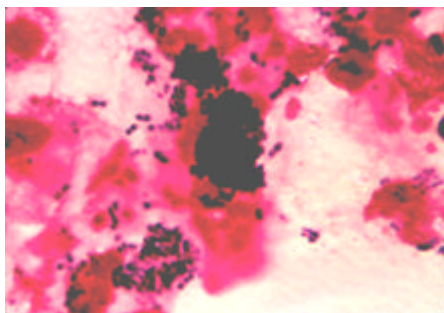
C. GRAM STAIN :

The Gram stain is utilized to identify bacteria, fungi, as well as Acanthamoeba.

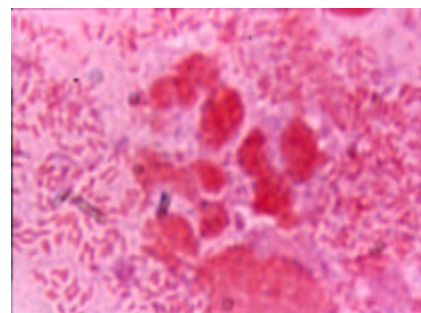
60 to 70% of bacteria can be identified by Gram stain.

Fungal filaments exhibit variability in their staining pattern. Yeasts stain violet in colour, and fungal filaments appear either as Gram negative (pink) or as faintly stained outline of fungus with unstained protoplasm.

Gram stained smears of corneal scrapings (100X magnification)



Plenty of gram positive cocci in pairs morphologically resembling *Streptococcus pneumoniae* are seen



Plenty of Gram negative bacilli are seen

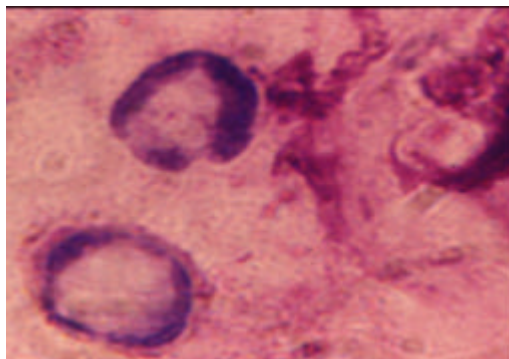
D. GIEMSA STAIN :

The Giemsa stain is mainly utilized to see the inclusion bodies of *Chlamydia trachomatis* in infected epithelial cells. Direct demonstration of viral antigens or viral products are possible by Giemsa stain cytology. Cytopathologic changes such as syncytial giant cells with Cowdry type A intranuclear acidophilic inclusions in Herpes Simplex Virus and Varicella Zoster Virus infections and intranuclear inclusions in adenovirus infections are demonstrable. Acanthamoeba cyst can be seen as a double walled cyst, the cell walls stained dark blue and cytoplasm stained blue. All bacteria are seen as dark blue bacilli or cocci. Inflammatory cells can be differentiated into polymorphs and different mononuclear cells.

The value of the Giemsa stain for distinguishing infectious from non-infectious keratitis by type of inflammatory cells has not been established. However, Giemsa stain cytology is an useful screening procedure to arrive at an aetiological diagnosis taking into account the type of inflammatory cells seen in the smear.

Corneal scraping :

Geimsa stained smear showing the typical double walled cysts of Acanthamoebae

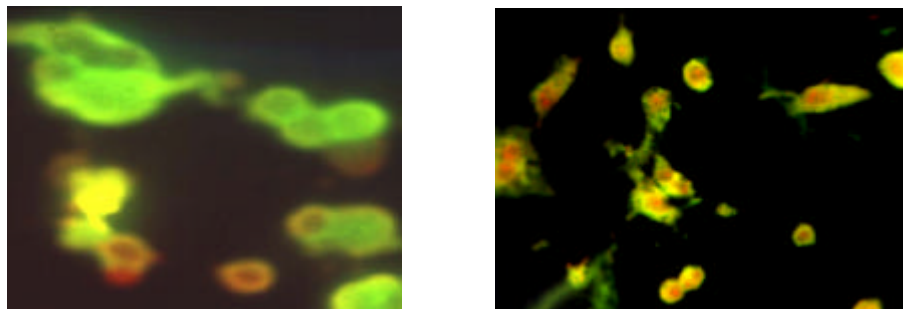


• OTHER SPECIAL STAINS :

- a) **Ziehl-Neelsen stain :** Is used for suspected Mycobacterial keratitis cases. The Acid fast bacilli are seen as red bacilli in a blue background.
- b) **Modified Ziehl-Neelsen stain** (with 1% Sulphuric acid as the decolouriser) : Is used to differentiate Nocardia species from Actinomyces. Nocardia is seen as red filamentous bacilli and Actinomyces is seen as blue colour filamentous bacilli.
- c) **Gomori Methenamine silver(GMS) stain :** Fungal filaments are seen in black colour against light green background.
- d) **Periodic Acid Schiff (PAS) stain :** Fungal elements take up Magenta colour.

E.. IMMUNOFLOURESCENCE STAINING

Imunoflourescence staining for detection of Herpes simplex virus –Positive



Imunoflourescence staining for detection of viral and Chlamydial antigens

With the availability of specific monoclonal and polyclonal antibodies against viruses and their antigens, immunodiagnostic methods allow detection of virus in the direct smears - like Herpes Simplex Virus (HSV) type 1 and 2 in viral keratitis cases, adenovirus in cases of epidemic keratoconjunctivitis. Monoclonal antibodies against outer membrane proteins of Chlamydia trachomatis are used to detect chlamydial antigens directly from the smears. These tests are commonly used for examination of smears and they are rapid, specific and sensitive. Exudates collected in buffers, and smears are used for EIA. Several EIA formats have been described for detection of this virus in specimens from cornea. . For detection of HSV in corneal ulcer, immunofluorescence is found to be highly reliable but results have to be considered with caution when patient is on antiviral therapy. Immunoflourescence on direct smears has been found to be more sensitive than culture for detection of HSV in corneal ulcers particularly in those which are on treatment with acyclovir. This is likely to be due to the presence of HSV antigens rather than replicating virus in the lesions.

F. ELECTRON MICROSCOPY :

Electron microscopy is not a common diagnostic procedure for rapid detection of viruses causing keratitis.

CULTURES :

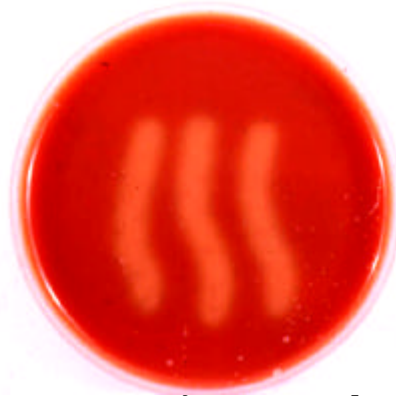
CULTURE FOR BACTERIA (AEROBIC AND ANAEROBIC BACTERIA) :

Culture plates are incubated in different environments. (aerobic/anaerobic/CO₂ atmosphere) as given in table. 1.

A daily monitoring of culture media is essential, and based on the standard procedures for description of colony morphology, selection of colonies for processing and antibiotic susceptibility testings are done. In general within 24-48 hours bacterial

growth is observed. An isolate is considered significant if it is consistent with the clinical signs and direct smear results, and if the the same organism is grown in more than one media, and or if the same organism is grown from repeated scrapings.

**Beta haemolytic colonies of *Pseudomonas aeruginosa*
on the inoculated "C" curves of corneal scrapings**



Culture for Mycobacteria

Lowenstein Jensen's medium

teria.

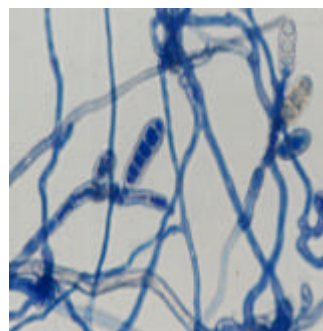
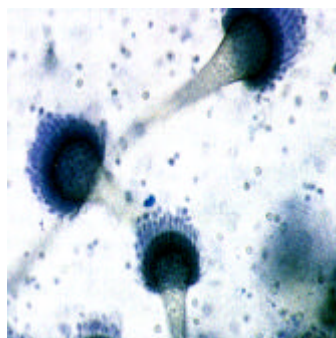
Kirchner's liquid medium is
of specimens like ocular specimens

ly for small quantities

CULTURE FOR FUNGUS:

Fungus culturing media - (Sabouraud's Dextrose Agar - SDA) for ocular specimens should not contain cycloheximide - an inhibitor of saprophytic fungi - since most fungi causing keratitis are due to saprophytes. Fungus grows within 24 hours to maximum of two weeks time - so prolonged incubation at 25°C is essential at least for two to three weeks before the culture is considered negative. The value of antifungal susceptibility testing for treatment of mycotic keratitis is not yet established.

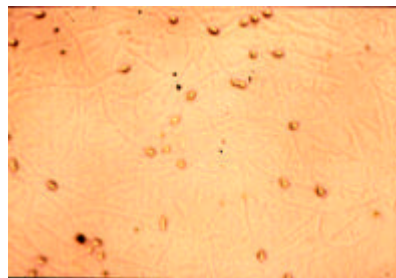
**Lactophenol cotton blue preparations of slide cultures of
Aspergillus fumigatus *Curvularia* species**



CULTURE FOR ACANTHAMOEBA :

Non nutrient agar (NNA) is the standard medium used with an overlay of *Escherichia coli* for the growth of *Acanthamoeba*. The specimen is simply touched to the surface of the plate without streaking or breaking the surface. Two plates may be inoculated for incubation at 25 and 37°C since some species do not grow at the higher temperature, and the plates are examined for trophozoites and cysts directly under the microscope. Trophozoites may be seen in 24 to 48 hours. They move and cover the entire plate surface on further incubation and turn into cysts. The plates should be observed for atleast 10 days.

Lawm mown appearance of the track of acanthamoebae trophozoites feeding on the *E.coli* on Non nutrient agar plate



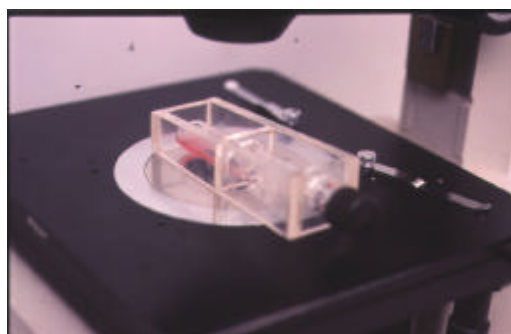
CULTURE FOR VIRUSES :

The most sensitive and specific method for diagnosis of HSV and Adeno virus infection is the conventional method of isolation and identification of viruses in cell cultures, which usually requires upto 2 to 4 weeks. This is more sensitive than direct methods described earlier for detection, because the virus content is amplified by growth in susceptible host system such as cell cultures. Both primary and established cell lines are extensively used for isolation and identification of HSV and adenoviruses.

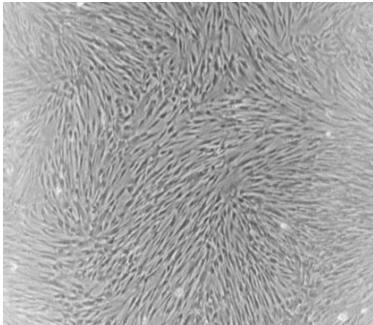
Shell Vial Technique



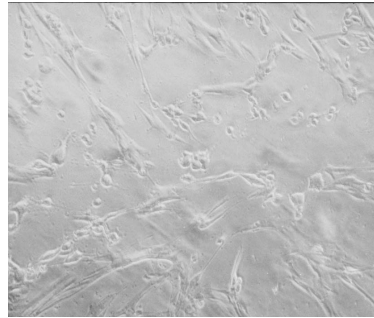
Conventional tube culture technique



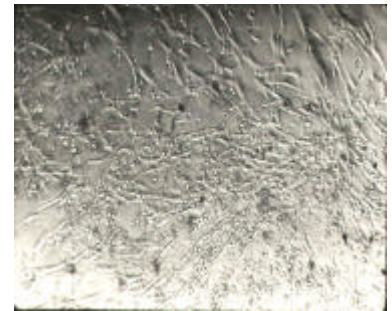
**Healthy fibroblast cell line
(WI 38 CELL LINE)**



CPE by Cytomegalovirus



**CPE produced by
Varicella Zoster virus**



Other viruses :

Mumps and Measles viruses cause keratitis and Mumps keratitis is almost always one of the ocular complications of the systemic disease. Immunofluorescence can be used to demonstrate the viral antigen in the conjunctival epithelial cells. Infection of the eye with the measles virus may result in mild epithelial punctate keratitis and rarely interstitial keratitis. Corneal ulceration may occur. Diagnosis is made by isolation of the virus. Molluscum Contagiosum and vaccinia also can cause severe keratitis rarely. Diagnosis is made by viral isolation, and humoral antibody response.

DIAGNOSIS OF CONTACT LENS ASSOCIATED KERATITIS :

Contact lenses, if present on the eye, should be removed aseptically and placed in sterile saline and sent to the laboratory, where they can be cultured by agar sandwich method. Fluid from the lens cases can be cultured on standard media such as blood agar, MacConkey agar, NNA and SDA. Microscopy of the lens deposit, centrifuged deposit of the lens care solutions may help detect the causative organism.

SEROLOGICAL METHODS OF DIAGNOSIS :

The traditional serological methods for diagnosis of corneal infections are generally of little value, since most of the infections are secondary to a primary infection elsewhere in the body.

NEWER METHODS IN THE DIAGNOSIS OF INFECTIOUS KERATITIS :

The introduction of new techniques such as immunochemistry, fluorescent microscopy, enzyme immuno assays, radioimmunoassays and molecular biologic techniques has led to the modification of the conventional techniques, for rapid identification of the various etiological agents of ocular infections within 1 to 6 hours. Most immuno assays are based on the availability of specific antisera against infectious agents based on hybridoma technology. Such monoclonal antibodies have been used in the diagnosis of viral, chlamydial and bacterial infections.

Recent introduction of nucleic acid hybridisation technique has revolutionized the field of diagnostic microbiology. This method not only detects the species of microorganisms but also the strain of the organisms, thus providing information on antibiotic susceptibility of the organism. Various diagnostic test kits are now being marketed for various diseases which are highly sensitive and specific. The availability of probe is increasing with time and this technique is going to have a tremendous impact on the rapid diagnosis of infectious keratitis and many other ocular diseases.

POLYMERASE CHAIN REACTION (PCR) :

PCR for detection of HSV has recently been used by several workers and is found to be a quick and sensitive method for detection of specific herpes virus in keratitis of unknown aetiology.

PCR is now routinely used to detect genomes of

- *Chlamydia trachomatis*
- slow growing *Mycobacterium tuberculosis*
- viruses like Herpes simplex virus ,
- Varicella Zoster virus .
- Adeno virus in our laboratory for corneal specimens

INFECTIOUS ENDOPHTHALMITIS (BACTERIAL & FUNGAL)

Endophthalmitis refers to the inflammatory process that involves the ocular cavity and adjacent structures. Infectious endophthalmitis is caused mainly by bacteria and fungi. Viruses and parasites as causative agents are very rarely

implicated. There is increasing recognition now that virtually any bacterium or fungus can cause endophthalmitis if introduced in sufficient quantities.

Classification :

Infectious endophthalmitis can be classified according to the mode of entry, type of etiological agent and location in the eye. Based on the mode of entry into the eye, endophthalmitis is exogenous when the microorganisms are introduced into the eye from the environment. Endogenous infection is caused by the haematogenous spread of organisms into the eye as a metastatic infection from an infected site elsewhere in the body. Exogenous endophthalmitis usually occurs either following surgery (post-surgical or post-operative endophthalmitis) or trauma (post-traumatic endophthalmitis) or may involve the intra-ocular contents in a generalized fashion. When the episclera participates significantly in the inflammatory process, a panophthalmitis is said to be present.

Post-operative endophthalmitis :

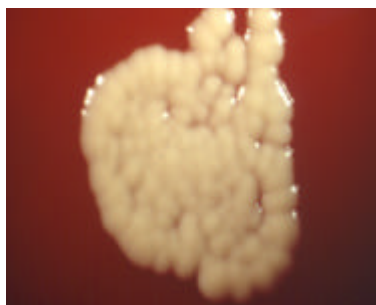
This is the most common form of endophthalmitis accounting for approximately 70% of infectious endophthalmitis. It may occur after any surgical procedure during which there has been communication between the interior of the eye and the external environment. The large majority of post-operative endophthalmitis follows cataract surgery since it is the most common ophthalmic surgical procedure performed.

Among the many potential sources of infection during surgery, the most common is the periocular flora of the patient. Indeed, around 75% of the conjunctival cultures from normal eyes harbor *S. epidermidis*, *S. aureus* and various streptococci. A similar pattern has been found in eyes with post-operative endophthalmitis. The role of external ocular bacterial flora in the pathogenesis of post-operative endophthalmitis has been proven by DNA studies done on *S. epidermidis* endophthalmitis. The periocular flora normally gain access into the eye

during surgery. Organisms may be carried into the eye as surface fluid refluxes through the wound during surgery. Additionally, an intraocular lens can become contaminated if it touches the ocular surface or with the air of the operating room. Epidemics have been reported following the use of contaminated irrigation solutions and lens implants. Based on the time of onset of symptoms after surgery, post-operative endophthalmitis is classified into acute and later onset endophthalmitis. Acute (2 to 7 days after surgery) post-operative endophthalmitis is usually fulminant and most often caused by *S. aureus* and Gram negative bacilli. Late onset post-operative endophthalmitis may fall into 2 groups.

Bacterial endophthalmitis, the most severe form of vision threatening ocular infection may follow surgery, trauma, bacterial keratitis or may be of endogenous origin. Several Gram positive and Gram negative bacteria including anaerobic bacteria cause endophthalmitis. The value of culture of vitreous and aqueous samples in diagnosis of infectious endophthalmitis is well established, but often negative cultures are encountered resulting in a clinical dilemma over the cause of the inflammation since it is not often possible to clinically differentiate other similar inflammatory conditions such as phacoanaphylactic uveitis and idiopathic postoperative inflammation from infection. A rational therapy on the use of antibiotics and steroids necessitates to determine whether the inflammation is infectious or sterile. Therefore, in such cases, an aetiological diagnosis is necessary.

**Colony of *Candida albicans*
on Blood agar plate**



**Colony of *Staphylococcus epidermidis*
on blood agar plate**



Chronic endophthalmitis is seen in patients who manifest the signs of inflammation late but in whom all evidence indicates that the organisms have actually been present within the eye since the surgical procedure. The organisms commonly associated are the relatively less virulent ones such as *Propionibacterium acnes*, *S. epidermidis* and fungi.

The infective agents enter the eye long after surgery (during the post-operative period) and develop endophthalmitis rapidly. The patient must have some avenue for the source of infection such as wound dehiscence or filtering bleb. The organisms commonly associated are *Haemophilus influenzae* and *Streptococcus* spp.

Aetiological agents :

The most common organisms responsible for post-operative endophthalmitis include mainly Gram positive bacteria accounting for 76-90% of cases followed by Gram negative bacteria (3-22%) and fungi (3-8%). Among the Gram positive bacteria, *S. epidermidis* is the causative agent in the majority of the cases (33-63%) . The other common Gram positive bacteria include *S. aureus*, *S. pneumoniae*, *S. viridans* and *S. pyogenes*. Among the Gram negative bacteria isolated, *Pseudomonas aeruginosa* is the most common, although others such as *Klebsiella pneumoniae*, *H. influenzae*, *Escherichia coli* and *Enterobacter aerogenes* have also been isolated.

Anaerobic bacteria are as prevalent as aerobic bacteria as normal commensal flora of the conjunctiva. A chronic, low grade, delayed and often recurrent post-operative granulomatous uveitis is the typical presentation of *P. acnes* endophthalmitis. These slow growing pleomorphic Gram positive bacilli become sequestered in the equatorial regions of the lens capsule, where they may not be accessible to routine culture of intraocular fluids. The most common bacteria isolated were *Propionibacterium* spp, *Bacteroides* spp and anaerobic streptococci. Post-operative fungal endophthalmitis is uncommon, but many different fungi often considered saprophytes or opportunistic

pathogens (Eg. *Cephalosporium*, *Paecilomyces*, *Candida*, *Aspergillus* or *Penicillium*) have been isolated.

Bleb-induced endophthalmitis :

Patients with surgically produced filtering blebs for glaucoma or blebs resulting inadvertently after intra-ocular surgery are susceptible to the development of endophthalmitis months or years after surgery. Bacteria associated with bleb-related endophthalmitis must be capable of penetrating intact conjunctiva overlying filtering blebs in order to enter the eye and hence caused by more virulent bacteria such as *Streptococcus* spp. and *H. influenzae*.

Post-traumatic endophthalmitis :

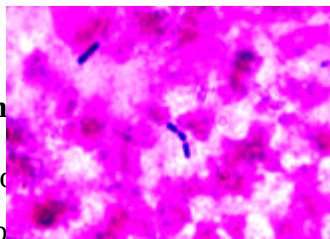
Endophthalmitis following penetrating eye injuries has a relative poor prognosis. This is due to the underlying eye trauma and the causation by more virulent bacteria such as *Bacillus* spp. as well as the attendant delay in diagnosis and treatment. These cases present difficult diagnostic and management issues, distinct from endophthalmitis occurring in other settings because of the co-existing ocular trauma.

The risk of endophthalmitis in cases of retained IOFB has been reported at between 6.9% and 13%. The largest of these series reported by Thompson et al. (492 IOB injuries) showed that the risk of endophthalmitis did not differ significantly for different types of foreign bodies – metallic – 7.2%, non-metallic - 6.8% and organic – 5.9% . Endophthalmitis occurring at a higher frequency in a rural as compared to a non-rural setting is attributed to a higher incidence of soil contamination in the rural areas.

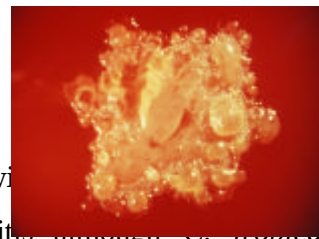
In addition to *Bacillus* spp, Gram positive cocci - both Staphylococci and Streptococci are more common than Gram negative bacilli and fungal isolates. Post-traumatic endophthalmitis can also be caused by anaerobes, commonly Clostridial

species such as Clostridial species such as *Clostridium perfringens*. Fungal infections are higher in injuries with vegetable matter. Eg. thorns, tree branches etc.

Cytospin intraocular fluid showing the typical gram positive bacilli (*Bacillus cereus*) from traumatic endophthalmitis



Colony of *Bacillus cereus* grown on blood agar plate

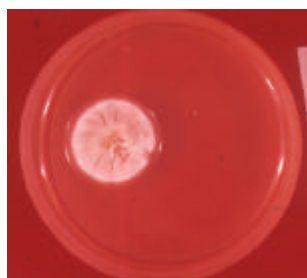


Endogenous

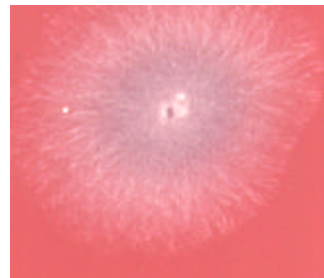
Endogenous is typically fungal, with *Candida* being responsible for 75-90% of endogenous endophthalmitis although *C. tropicalis*, *C. stellatoidea* and *C. krusei* have also been implicated. Second in frequency to *Candida* is *Aspergillus* spp. of which *A. fumigatus* dominates, but *A. flavus* has also been found. Various other saprophytic, opportunistic and pathogenic fungi including *Sporothrix schenckii*, *Cryptococcus neoformans*, *Coccidioides immitis* and *Mucor* have been reported in isolated cases.

Fungal colonies grown right on the inoculum on blood agar plates from the intraocular specimens of fungal endophthalmitis patients

***Aspergillus flavus*
On Blood agar**



***Fusarium* species
On blood agar**



Endogenous bacterial endophthalmitis is very rare. Until 1940s, *Neisseria meningitidis* accounted for more *S. pneumoniae* and *S. aureus*. After 1945,

infection with other bacterial pathogens, especially Gram negative bacilli (*E. coli*, *Pseudomonas* spp. and *Proteus* spp.) began to occur more frequently. In 1986, Greenwald and associates reported that *B. cereus* had become the most frequent cause of endogenous bacterial endophthalmitis.

Laboratory diagnosis :

Determination of whether the endophthalmitis is sterile or infectious, is to extreme importance. If infectious identification of the causative organism and the performance of susceptibility studies on the isolated bacterium is mandatory. A prompt and accurate etiological diagnosis of suspected endophthalmitis is essential for the appropriate and timely treatment which is central for a successful visual outcome. Timely action has to be taken to obtain ocular fluid for microbiological studies. The vitreous fluid (VF) is most likely specimen to yield the infecting organism even if the surgical procedure penetrating trauma or focus of inflammation such as bleb associated endophthalmitis is confined to the anterior segment.

Collection of intraocular specimens :

Uncontaminated VF is aspirated by a syringe connected to the suction port of the vitreous cutter at the beginning of vitrectomy. A sterile disposable needle is fixed to the syringe, the air in it expelled carefully without causing aerosis and the needle is capped with a sterile rubber bung and sent to the laboratory immediately. AH samples (150-200µl) are collected aseptically in a tuberculin syringe with a 30G needle.

Vitreous aspirate transport tube with a rubber cork inside.



After the air in the syringe is expelled to prevent inactivation of anaerobic bacteria, the needle is fixed onto a sterile rubber bung and placed in a sterile large test tube container, which is immediately transported to the laboratory. Specimens should be ideally processed within 15 – 30 minutes after collection. Transport media of the conventional kind have no place in the transport of these intraocular specimens.

Microbiological investigations :

Cultures and smears for detection of bacterial and fungal agents are carried out on the intraocular specimens as follows Culture : Inoculation of media for culture of bacteria and fungi is to be carried out first, because the number of organisms are likely to be low. The AH and VF being normally sterile fluids, do not require selective media for culture. For culture, a variety of media are included for the favorable growth of aerobic and anaerobic bacteria and fungi. However, the number of media included depends on the volume of the sample available. For inoculation onto solid media, 1-2 drops of the specimen is expressed through the needle onto the agar plate. The material is not spread with a loop to reduce the possibility of contamination. Liquid media are also inoculated with 2 - 3 drops. The culture media used for blood agar – BA (incubated aerobically at 37°C), Brucella blood agar – BBA (incubated 37°C anaerobically) chocolate agar – CA (incubated 37°C in an atmosphere of 10% CO₂) and liquid media - Brain heart infusion broth (BHIB) and Robertson cooked meat medium (RCM) / thioglycollate broth. Specimen inoculated onto Sabouraud dextrose agar (SDA) is incubated at 25°C. If cultures on BA, CA and BHIB show no growth at the end of 48 hours, they are placed at 25°C for another 7 days for the growth of fungus. The cultures for anaerobic bacteria are incubated for up to 2 weeks. The isolated bacteria and fungi are then identified by standard methods. The antibiotic susceptibility testing of the aerobic isolates is done by the standard Kirby-Bauer disk diffusion technique.

Direct Smear studies :

Three to four smears from the left over AH/VF specimens are made either by placing a dropt or two of the AH/VF onto clean glass slides and air drying them or preferably by concentrating the specimen using the cytopspin to obtain a uniform layer of flattened, well preserved cells, that are particularly well suited for cytological examination. The air dried smears are fixed in 95% methanol for 5 minutes prior to staining. The recommended stains are KOH or KOH – Calcofluor white preparation (if fluorescence microscope is available) for detection of fungus, Gram stain for bacteria and Giemsa stain for cytology. Microscopy, although frequently negative is helpful in the rapid discrimination between bacteria and fungi.

The criteria laid down to consider the isolated bacterium or fungus as the causative agent are it growth from VF / AH on two or more of the inoculated media or its growth on a single medium correlating with direct smear findings or repeated isolation of the same organism from two or more intraocular specimens of the patient.

Emergency surgeries for an open globe are performed at odd hours and these standard culture media may not be immediately available. In such cases, the specimens can be inoculated directly into standard culture media may not be immediately available. In such cases, the specimens can be inoculated directly into standard blood culture bottles in the operation theatres. Such inoculation can be performed by the surgeons immediately in the theatre. This minimizes handling of the specimen thereby reducing contamination and can be readily performed at nights and weekends and when fresh culture plates are inaccessible. The disadvantages of this method is that it is less sensitive for isolating *H. influenzae* and *P. acnes*. In spite of this drawback, inoculation of blood culture bottles may still be useful adjunct and simple alternative when the specimen processing techniques are not readily available.

Microbiological investigations of non-ocular specimens :

Culture of non-ocular specimens is very significant in the diagnosis of endogenous endophthalmitis. As an important first step, blood cultures should be obtained in all patients. Cultures of other non-ocular sites should also be obtained depending on the systemic symptoms of the patient. Cultures of the throat or sputum cultures in case of respiratory tract infection, stool cultures in case of respiratory tract infection, stool cultures in patients with gastro-intestinal infection, cerebrospinal fluid (CSF) in patients in whom meningitis cannot be ruled out should be done. Other occult sites of infection such as bone marrow, liver abscesses should be aspirated and cultured as they are responsible for an increasing number of intraocular infections.

Before the needle is fixed into the rubber cork, all air should be expelled from syringe. The needle should be fixed into a sterile rubber cork and placed in a large test tube which is provided in the operation theatre or available in Microbiology laboratory. sent immediately to the laboratory. This method helps in maintaining anaerobic bacteria viable. Anaerobic organisms are likely to be inactivated in 30-60 minutes even if air bubble is not present in the syringe.

PROCESSING OF SPECIMENS IN THE LABORATORY :

Laboratory procedure

It is essential inoculation of media is done first because as the number of organisms are likely to be low, every chance is given for them to multiply. Smears are not to be done first. Bacteria which are very few in number are difficult to be made out in smears. Bacterial cultures are done for both aerobic and anaerobic organisms and incubated at 37°C.

Aerobic

Anaerobic

Blood agar

Brucella blood agar

Chocolate agar

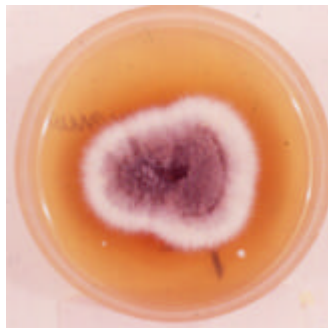
MacConkey agar

Brain heart infusion Broth - Thioglycollate broth/Robertson's medium

Culture for fungus :

- Sabouraud dextrose agar in small petridish
- α in test tube slants covered with cotton plug is used; incubate at 25°C.

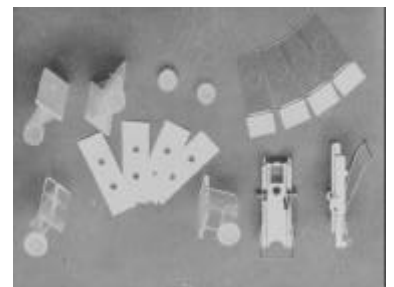
Fusarium species grown on Sabouraud's Dextrose agar plate with the typical diffusing pigment



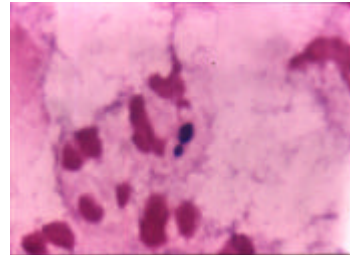
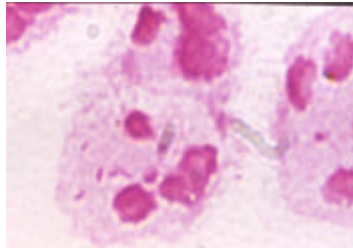
Direct Smears :

- a) 3-4 smears are made by cytospin and fixed in methyl alcohol.
- b) The smears are stained by : Gram stain, Giemsa stain, Gomari's Methanamine silver stain and a KOH & Calcofluor preparation is made for fungus.
- c) If sufficient material is not available smears may be made directly on to the slides and stained as above.

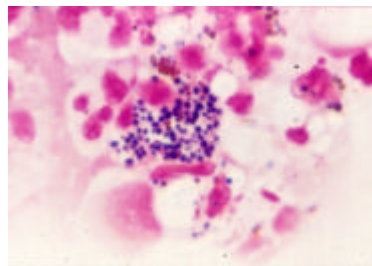
CYTOSPIN MACHINE AND THE ACCESSORIES



**Cytospined Gram stained smears of intraocular fluids showing
Intracellular Gram negative bacilli Gram positive budding yeast cell**



Gram positive cocci in clusters



Molecular biological techniques :

Culture of intraocular specimens is considered as the gold standard in the diagnosis of endophthalmitis. Nevertheless, even under the most appropriate care, traditional microbiological methods yield positive results in only 60-70% of the clinically typical cases of endophthalmitis. Our experience over the last 9 years also show a similar sensitivity of the routine methods (data unpublished). Prior antibiotic therapy, small number of organisms in the samples, possible localized nature of infections in the lens capsule and fastidious growth requirement of the offending organisms are some of the reasons that have been attributed to the organism not being recovered in roughly around 30-40% of the cases. In such cases, the most appropriate antibiotic coverage is not discernible and an aetiological diagnosis is very essential for a rational therapy. Additionally there may be a delay of several days before the cultures are interpreted. Fortunately, molecular techniques such as polymerase chain reaction (PCR) have come to the rescue. PCR, as a specific, sensitive and rapid technique in the identification of the pathogen in the clinical specimen has been developed extensively over the past decade. While it is primarily

used basic microbiological research, its value as a clinical tool is being increasingly recognized.

PCR can identify the offending microbe in less than 24 hours after the specimens are obtained. In a study carried out at our center too, we evaluated these primers on both AH/VF of patients with endophthalmitis and found it to be extremely sensitive in detecting the bacterial aetiology. It was felt that many of the culture negative endophthalmitis cases could be due to *P. acnes*. Therefore, PCR with primers specific for *P. acnes*, was also done on specimens which were smear and culture negative but showing PCR positivity for eubacterial genome.

It has been postulated that DNA sequencing of the universal nested PCR product may allow identification of the causative organism in a number of culture negative cases. PCR has also been evaluated in the diagnosis of fungal endophthalmitis using broad range primers as well as primers specific for *C. albicans*. Thus detection of bacterial or fungal DNA by PCR in intraocular specimens will prove as a useful means of diagnosing endophthalmitis. It will greatly facilitate management decisions when conventional culture is negative.

PCR THERMAL CYCLER TRANSILLUMINATOR HYBRIDIZATION OVEN



Gel photograph showing the 470bp amplified Product

Dot blot PCR- Hybridization technique using PCR amplified Product of eubacterial genome to differentially identify the gram positive and gram negative bacterial genome



OCULAR PARASITIC DISEASES

GENERAL CONSIDERATIONS :

Study of diseases in man caused by organism belonging to animal kingdom is called parasitology. Unicellular organism such as amoeba, malarial parasite, *Toxoplasma gondii* etc., cause infection in man. Metazoans like tapeworms and roundworms also cause infections in man.

INTRAOCULAR PROTOZOAL INFECTIONS OF EYE

Toxoplasmosis : Caused by *Toxoplasma gondii*

This protozoan has a life cycle in Cat : sexual life cycle in the epithelium of intestine Man and other animals; asexual life cycle in tissues including eye. Diagnosis is made by detecting antibodies to toxoplasma in patients serum. Disease of the eye and brain can occur as congenital infection.

HELMINTHIC INFECTIONS

Cysticercosis :

This is caused by *Taenia solium* which is tapeworm causing intestinal infection of pig. Man passes egg in his faeces. These eggs ingested by pig larva is liberated in stomach pass into the circulation and may lodge in muscles, meninges and eye and form a cyst called cysticercus cellulosae. Man ingests infected pork and develops taeniasis with the development of adult *Taenia solium* worm in the intestine. The eggs may regurgitate in to

the stomach from the intestine and larva may be liberated in the stomach pass in to the circulation and lodge in muscles, meninges and eye and form cysticercus cellulosae. Thus man can also develop larval forms in his tissues. Diagnosed by histopathology examination of the biopsy tissue. Serology by ELISA also can be used for diagnosis.

Echinococcosis: Hydatid disease

Caused by larva of *Echinococcus granulosus* - dog tape worm. Larval form develops in man and ungulates - sheep, buffalo, camel and deer .Egg (ovum) is liberated by adult tapeworm in the dogs intestine and passed in the faeces. The egg, when ingested by man or the ungulates hatch in the intestine, larva is liberated. Larva enters into circulation through intestinal wall and then lodge in various organs including eye. It grows into a cyst in which many more larval forms are developed. If dog ingests larva the adult form develops in its intestine, then completing the life cycle. Disease of eye often can be diagnosed by biopsy.

Toxocariasis :

Round worm, *Toxocara canis*, normally occurs in the intestine of pup. The egg liberated is passed into the faeces. If man ingests this egg, larva liberated from it, may pass into circulation and lodge itself into any organ including eye. This migration of larva in the unnatural host often caused the condition called larva migrans(Toxocariasis). Diagnosis is done by finding antibody in the serum against *Toxocara* larval antigen.

Other Parasitic diseases :

Entamoeba histolytica, malarial parasite, larval stages of filarial worm (*Wuchereria bancrofti*) and other helminths may cause eye diseases. Diagnosis is made by detecting antibodies in patients serum. Disease of the eye and brain can occur as congenital infection.

Ocular microbiology remains an applied science The advancements in molecular biology and the newer technologies pave way for better understanding of ocular diseases Advances in the field of infections diseases are rapid. The developments

have made major contributions in the control and probably even eradication of many types of eye infections