

Major Review

Microbiology for general ophthalmologists

ABSTRACT

Since ocular surface infections are very common in everyday ophthalmic practice, every ophthalmic personnel are familiar with their etiologies and the basic techniques for the diagnosis.

Keywords: Culture and sensitivity, Gram's staining, KOH staining, microbial flora

INTRODUCTION

Ocular surface infections are very common in everyday ophthalmic practice. Hence, every ophthalmic personnel are familiar with etiologies of these infections and the basic techniques for their diagnosis.^[1] Although eyes are relatively impermeable to microorganisms, intraocular infections result from trauma, surgery, or systemic disease.^[2] While evaluating a patient with infection, it is of great help to be familiar with the normal microbial flora of the human eye.^[1]

NORMAL MICROBIAL FLORA OF EYE

Few microorganisms are present normally in the conjunctival sac.^[3]

Aerobic

- **Gram-positive cocci**
 - *Staphylococcus epidermidis*
 - *Staphylococcus aureus*
 - *Micrococcus*
 - *Streptococcus pyogenes*
 - *Streptococcus pneumoniae*
 - *Streptococcus viridans*
- **Gram-negative cocci**
 - *Moraxella catarrhalis*
- **Gram-positive bacilli**
 - *Corynebacterium* species
- **Gram-negative bacilli**

- *Haemophilus influenzae*
- *Klebsiella* sp.
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Moraxella* sp.

Anaerobic

- *Propionibacterium* sp.
- *Peptostreptococcus*
- *Bacteroides* sp.
- *Lactobacillus* sp.
- *Clostridium* sp.

Fungus

These are transient and are those found in the environment.

MICROBIOLOGY OF OCULAR SURFACE INFECTIONS

To get a better result with microbiologic examination, the ophthalmologist must inform the laboratory about the type of specimen, expected pathogens, and specific antibiotic susceptibilities that must be tested.^[4] For diagnosing unusual pathogens, special planning is required.^[4]

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OCULAR SPECIMEN COLLECTION

Peculiarities of ocular specimen:^[3]

1. Small amount of material
2. Number of organisms very less
3. Most of the time patients are on topical medication while taking specimen
4. Specimen may have nonviable organisms
5. Most of the time liquid media has to be used
6. Frequently fastidious organisms are encountered; therefore, enriched media are necessary.

- Moistened swab rubbed over conjunctival sac from medial to lateral and then back
- Swab is directly inoculated onto blood agar (aerobic incubation), chocolate agar, and brucella blood agar.

Conjunctival scraping is rarely taken and used to identify chlamydia and viruses.^[3]

INFECTIVE KERATITIS (MICROBIAL KERATITIS)

It is an important cause for corneal blindness. A wide range of

Conjunctivitis

Conjunctivitis is usually diagnosed by clinical features.^[3]

Conjunctival specimen

Soft-tipped applicators with plastic handles and heads composed of cotton, Dacron, or calcium alginate are used for conjunctival specimen collection.^[3,4] Calcium alginate swabs produce a higher yield of bacterial organisms.^[4]

Steps

Following are the steps of conjunctival specimen collection [Figure 1].^[3]

- Patient looks up
- Lower lid pulled down

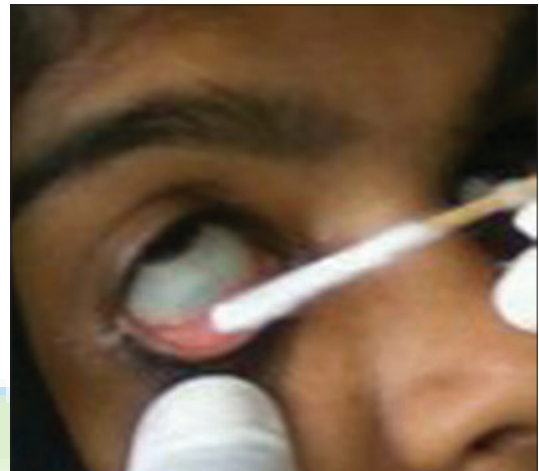


Figure 1: Conjunctival swab collection

Table 1: Causative organisms and its clinical features

Organism	Lesion
Gram-positive bacteria ^[3]	Discrete, well-defined, small abscess like lesion
Gram-negative bacteria ^[3]	Diffuse rapidly spreading necrotic lesions
Specific bacterial ulcers	
<i>Pseudomonas aeruginosa</i> ^[3]	Rapidly spreading ulcer. Acute pain and watering, scleritis
<i>Streptococcus pneumoniae</i> ^[3]	Rapidly spreading ulcer. Acute pain and watering. Infectious crystalline keratopathy
<i>Staphylococcus aureus</i> and <i>Moraxella</i> species ^[3]	Indolent corneal ulcers
<i>Corynebacteria</i> ^[3]	Diffuse epithelial haze, stromal necrosis, melting
<i>Listeria</i> ^[3]	Ring ulcer with severe anterior uveitis
<i>Clostridium</i> ^[3]	Frothy bullous keratopathy due to gas formation
<i>Propionibacterium acnes</i> ^[3]	Indolent ulcer with stromal abscess
<i>Nocardia</i>	Cracked windshield appearance
Pneumococcal or <i>Pseudomonas</i> ^[3]	Hypopyon
Pneumococcal or herpes simplex virus ^[3]	Hemorrhagic hypopyon
Fungal ulcers ^[3,6]	Insidious onset and indolent course, starts in the midperiphery of cornea in the interpalpebral area and spreads toward the center. Dry raised lesion, feathery margins. Moderate hypopyon or cheesy hypopyon, endothelial plaque, and posterior corneal abscess frequently. Satellite lesions and immune ring
Pigmented (dematiaceous) fungi ^[3]	Pigmented ulcers (chromomycosis)
<i>Acanthamoeba keratitis</i> ^[3,4,7]	Very severe pain. Ring of stromal infiltration at midperiphery of the cornea with intact gray or hazy epithelium. Superficial punctate keratitis, small dendrites, subepithelial keratopathy, satellite lesions, radial keratoneuritis. Limbitis
HSV Type I ^[3,4]	Epithelial, stromal, endothelial lesions. Epithelial - dendritic keratitis, marginal herpetic keratitis, geographic or amoeboid ulcers, or trophic ulcers Stromal - superficial keratitis with epithelial lesions, disciform keratitis, diffuse stromal keratitis Endotheliitis Limbitis

HSV – Herpes simplex virus

microbial agents cause corneal infections. A rapid etiological diagnosis has an important role in initiating an aggressive and targeted treatment. Clinical features of infectious keratitis may help in the initial etiological diagnosis [Table 1].

Instruments used for corneal specimen collection are spatulas, Jeweler's forceps, and surgical blades.^[3,4] Cotton-tipped applicators are used in less aggressive infections, and less distinct areas of involvement over a large area require culture.^[3,4]



Figure 2: Corneal scraping

Steps

Corneal specimen is collected as follows [Figure 2].^[3]

- Anesthetize the eye (avoid tetracaine which has antibacterial action), clean the eyelids and conjunctival sac
- Scraping taken from the edge and the base of the corneal lesion under slit lamp or operating microscope
- Material is inoculated directly onto various culture media
- Make smears on glass slides, mark with wax pencil on reverse side, and stain with various stains
- If any delay for inoculation store the specimen at -20°C
- In case of therapeutic keratoplasty, corneal button or corneal biopsy tissues are cut into small bits in sterile conditions and inoculated on various culture media
- Excessive scraping leads to corneal scarring and long-term poor visual acuity.^[6]

Transport media

Direct inoculation of specimen to the culture media is the best but may not be possible always.^[4] Many transport systems for supporting bacteria, virus, and chlamydia before direct inoculation on proper isolation media are available.^[4] For example, Amies transport medium.^[8]

Common stains and tests

Commonly used stains are Gram stain, acridine orange,

Table 2: Staining characteristics and microscopic appearance of causative pathogens

Stain	Organisms	Appearance
Gram stain ^[3,6]	Bacteria	Gram-positive-violet [Figure 3] Gram-negative-pink
	Fungi	Fungal filaments exhibit variable staining pattern. Yeasts - violet [Figure 4]. Fungal filaments - Gram-negative (pink) or faintly stained outline of fungus with unstained protoplasm Sensitivity 36%-50%
KOH wet preparation ^[3,5,6]	<i>Acanthamoeba</i>	Cyst-Gram-positive
CFW stain wet preparation ^[3,7]	Fungal filaments	Refractile and prominent. Sensitivity - 61%-94% specificity - 91%-97%
	Fungal filaments <i>Acanthamoeba</i>	Bright white, sensitivity 98.3% (CFW + KOH)
Giemsa stain ^[3,4]	Chlamydia trachomatis in infected epithelial cells	Inclusion bodies and type of cellular response
	Viral antigens or viral products	Eosinophilic inclusion bodies and type of cellular response
	<i>Acanthamoeba</i>	Cyst-double walled cyst, the cell walls stained dark blue and cytoplasm-stained blue
	Bacteria	Dark blue bacilli or cocci
	Actinomycetes	Long thin filaments with branches or long thin rod
Ziehl-Neelsen stain ^[3]	Acid-fast bacilli [Figure 5]	Red bacilli in a blue background
Modified Ziehl-Neelsen stain (with 1% sulfuric acid as the decolourizer) ^[3]	<i>Nocardia</i>	Red filamentous bacilli
	Actinomycetes	Blue filamentous bacilli
GMS stain ^[3]	Fungal filaments	Black color against light green background
	Fungal elements	Magenta color
PAS stain ^[3]	Fungus	Sensitivity of 85%, specificity of 90%-91%
Lactophenol cotton blue ^[3]	Fungus [Figure 6]	
Immunofluorescence staining ^[3]		Rapid, specific, and sensitive
Viral and chlamydial antigens		
Immunoperoxidase ^[7]	<i>Acanthamoeba</i>	

GMS – Gomori methenamine silver, PAS – Periodic acid-Schiff, CFW – Calcofluor white, KOH – Potassium hydroxide

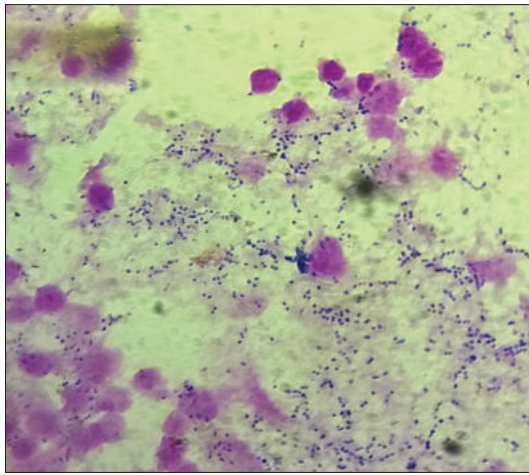


Figure 3: Gram staining - Pneumococci

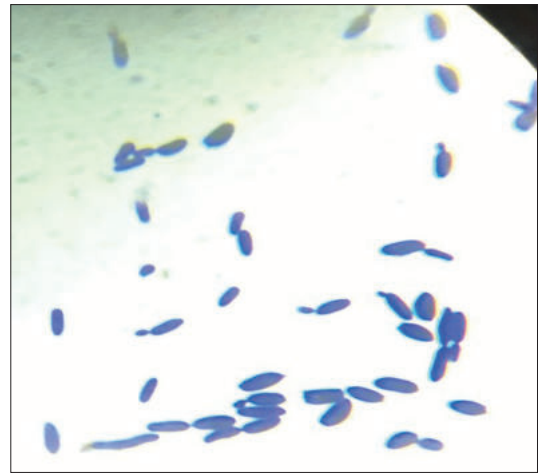


Figure 4: Gram staining - *Candida*

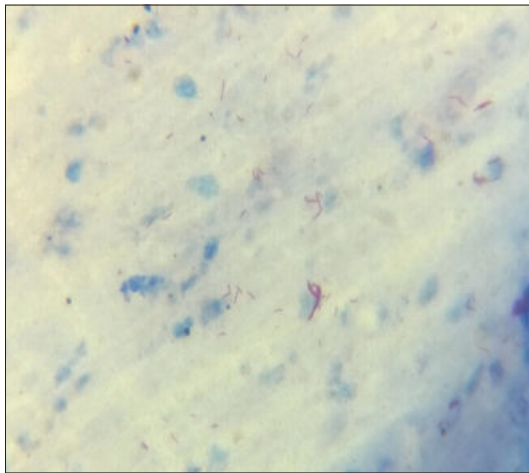


Figure 5: Acid-fast bacilli

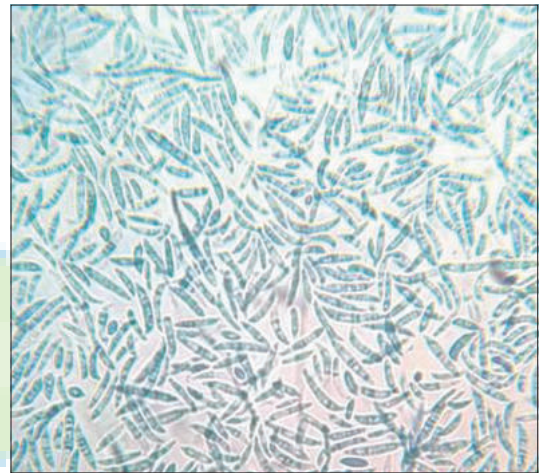


Figure 6: Lactophenol cotton blue staining - *Fusarium*

calcofluor white, and acid-fast, Giemsa stain.^[4] All bacteria isolated from the cornea should be considered as pathogens and tested for antibiotic susceptibilities.^[4]

PROCESSING AND INTERPRETATION OF DIRECT SMEARS

Smears are interpreted by staining methods [Table 2].

CULTURE OF CORNEAL SPECIMEN

Routine media for isolating bacteria from the cornea, conjunctiva, and eyelids are sheep blood agar, chocolate agar, and mannitol salt agar, enriched thioglycollate liquid medium.^[4] Inoculated culture media should be kept and examined for at least 5 days [Table 3].^[4] The criteria, to consider the growth on culture to be significant, are if growth is confluent (more than 10 colonies) on the site of inoculation on the solid media, or the organism is seen in the smears, or if the same organism is grown in more than one medium.^[8]

Corneal biopsy

Indications:

- Poor response to treatment
- Repeated negative culture in the presence of clinical feature strongly suggestive of infectious process
- Deep stromal infiltrate with uninvolved superficial layers.

Corneal biopsy can be taken under slit lamp or operating microscope. Under topical anesthesia, a trephine or a blade is used to excise a small piece of stromal tissue. Femtosecond laser can also be used as an alternative. A 7-0 or 8-0 vicryl or silk suture can be passed through the abscess without disturbing the overlying epithelium. The organism may attach to the fibers of the suture which can be cultured. Another option is to take biopsy under a lamellar flap.

Uncommon organisms

Mycobacteria

They are fastidious organisms and require long incubation periods for isolation.^[4] Mycobacteria keratitis is due to

Table 3: Culture media used for isolating organisms and their appearance

Organism	Culture media	Appearance
Bacteria ^[3,6]	Blood agar [Figure 7], Brucella blood agar, chocolate agar, MacConkey agar, brain heart infusion broth, thioglycolate broth/Robertson cooked meat medium, Mannitol salt agar	Description of colony morphology, selection of colonies for processing, and antibiotic susceptibility testing. 24-48 h - minimum period
Mycobacteria ^[3]	Lowenstein Jenson's medium, Kirchner's liquid medium	Small quantities of specimens
Fungus ^[3,4,6]	Sabouraud's Dextrose agar without cycloheximide [Figure 8], blood agar, chocolate agar	Fungus grows within 24 h to maximum of 2 week time - prolonged incubation at 25°C is essential at least for 2-3 weeks before the culture is considered negative. Molds have a fuzzy appearance and yeasts form colonies similar to bacteria
<i>Acanthamoeba</i> ^[3,4,7]	NNA with <i>Escherichia coli</i> overlay. Axenic liquid medium and cell culture	Two plates are inoculated for incubation at 25°C and 37°C (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 24-48 h. On further incubation, it turns into cysts. The plates should be observed for at least 10 days
Viruses ^[4]	HSV, adenovirus mumps, measles, molluscum contagiosum, and vaccinia	The most sensitive and specific method, - isolation, and identification of viruses is cell cultures, which usually requires up to 2-4 weeks (by cytopathic changes)

NNA – Nonnutrient agar, HSV – Herpes simplex virus

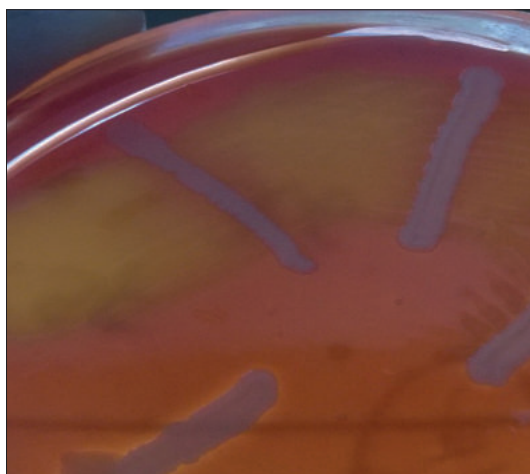


Figure 7: Blood agar - Pneumococcal growth



Figure 8: Sabouraud dextrose agar - Candida growth

rapidly growing organisms such as *Mycobacterium chelonae* and *Mycobacterium fortuitum* which grows on routine culture media such as blood agar or chocolate agar.^[4] Acid-fast staining and inoculation in Lowenstein-Jensen media can be used for mycobacterial isolation.^[4]

Nocardia* and *Actinomyces

These are less frequent ocular pathogens.^[4] *Nocardia* can be cultured on routine bacterial culture media.^[4] *Actinomyces* requires extended growth period, and cytologic examination of corneal specimen by Giemsa stain is diagnostic test (long thin filaments with branching).^[4]

Microsporidia

The stains commonly used to identify *Microsporidia* are modified trichrome, Giemsa, Gram, periodic acid-Schiff, Grocott's methenamine silver, calcofluor, and acid-fast methods.^[4]

Chlamydia

Cell culture isolation, Giemsa stain, and polymerase chain reaction are the methods used to detect chlamydia from ocular samples.^[4]

Rare pathogen that cannot be easily cultured need to be tested in special laboratories or at facilities researching the particular disease.^[4]

ANTIBIOTIC SUSCEPTIBILITY TESTING

It is performed on fast-growing bacterial isolates to assess whether an antibiotic will be successful in therapy or not.^[4] The disc diffusion method and minimum inhibitory concentration (MIC) methods are used to determine antibiotic susceptibility *in vitro*. In disc diffusion method, paper disks impregnated with a set amount of antibiotics is placed on a lawn of bacteria and incubate for 24 h,

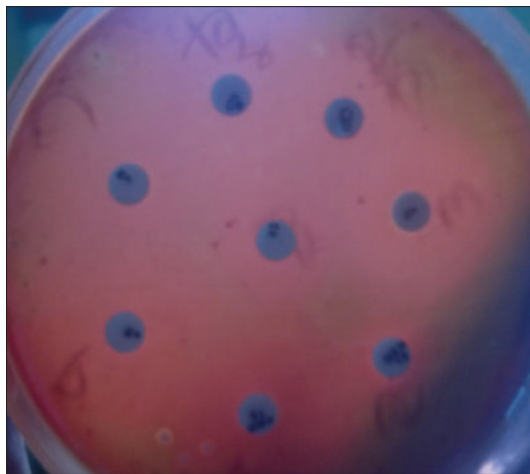


Figure 9: Minimum inhibitory concentration method for susceptibility

then zones of inhibition are measured and compared to predetermined zone standards [Figure 9].^[4] MIC methods include broth dilution, agar dilution, and E-tests.^[4] With these methods, MIC of antibiotic required to inhibit bacteria is determined and compared to predetermined standards that represent susceptibility.^[4] Ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin, gatifloxacin, oxacillin, cefazolin, vancomycin, gentamicin, and tobramycin are the commonly tested antibiotics in corneal infections.^[4]

DIAGNOSIS OF CONTACT LENS-ASSOCIATED KERATITIS

In contact lens-associated keratitis, the lens, container, and the solution have to be examined and cultured.^[3,6] Contact lenses should be removed aseptically, placed in sterile saline, and can be cultured by agar sandwich method.^[3] Contact lens solution can be cultured on standard media such as blood agar, MacConkey agar, nonnutrient agar, and Sabouraud dextrose agar.^[3] The lens deposits and centrifuged deposit of the lens case solutions can be examined under microscope to detect the organism.^[3]

SEROLOGICAL METHODS OF DIAGNOSIS

The serological tests have little value in the diagnosis of corneal infections.^[3]

Newer methods in the diagnosis of infectious keratitis

The newly introduced techniques such as immunochemistry, fluorescent microscopy, enzyme immunoassays, radioimmunoassays, and molecular biologic techniques are highly specific and sensitive [Figure 10].^[3] They help to identify the various etiological agents of ocular infections within 1–6 h.^[3]

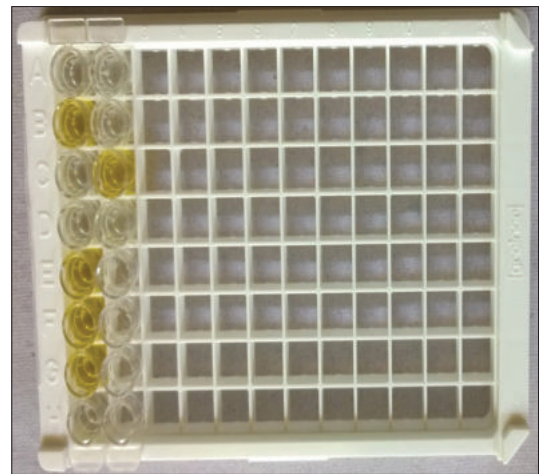


Figure 10: ELISA test

Polymerase chain reaction

This test is rapid and requires small sample but expensive and not readily available. It is used for detection of chlamydia trachomatis, slow-growing *Mycobacterium*, viruses such as herpes simplex virus, varicella zoster virus, adenovirus, *Acanthamoeba*, and *Fungi*.^[3-7,9]

DNA genotyping

This is used for rapid species identification of the organism including fungi.^[5]

Confocal microscopy

It is the instant imaging of organisms that are larger than a few micrometer such as *Acanthamoeba* cysts^[7] and fungal hyphae^[5,6]

H1 nuclear magnetic resonance spectroscopy

This test is used to identify *Acanthamoeba*.^[7]

Nucleic acid hybridization technique

This test used to identify both species and strains of the organism.^[3]

Smartphone-based digital imaging

This is recently used in the diagnosis and follow-up of keratitis.^[3]

Prolonged use of topical antibiotics results in change in normal microbial flora with introduction of fungi and antibiotic-resistant forms.

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Conflicts of interest

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