Role of Microbiology in the Diagnosis of Corneal and Conjunctival Infections

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15.1 Introduction

Infectious conjunctivitis and keratitis may be caused by several groups of organisms including bacteria, viruses, fungi, and parasites. Viruses cause up to 80% of conjunctivitis [1–4]. Many cases may be misdiagnosed as bacterial conjunctivitis and the accuracy of clinical diagnosis is 50% compared to laboratory confirmation [5]. Up to 90% of acute viral conjunctivitis are caused by adenoviruses with herpes simplex virus, varicella zoster virus, enteroviruses, coxsackieviruses, and other viruses [6]. Conjunctivitis has been described in viral infections such as measles, mumps, and Zika virus infection [7, 8]. Apart from viruses, several species of bacteria such as *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Haemophilus influenzae, Streptococcus pyogenes,* and *Staphylococci* are associated with conjunctival infection, especially in children. Concomitant with urethritis *N. gonorrhoeae* can cause bilateral purulent conjunctivitis with or without corneal involvement in adults. Isolated fungal infections of the conjunctiva are rare. Earlier classified with protozoa Microsporidia are now considered a parafungus and have been reported to be associated with keratoconjunctivitis that is clinically similar to adenovirus infection [9]. *Chlamydia trachomatis* is an obligate intracellular parasite that is responsible for several forms of conjunctivitis such as trachoma and inclusion conjunctivitis (adult and neonatal). Among the parasites, *Onchocerca volvulus* may cause conjunctival and corneal lesions by invasion and subsequent death of the microfilariae [10].

As listed in Table 15.1, various types of bacteria can potentially cause keratitis. The relative frequency of different bacteria as causative agents in keratitis may be geographical location based as well as risk factor and host dependent. The incidence of pneumococcal keratitis, which is commonly associated with chronic

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Table 15.1 Various genera of bacteria associated with bacterial keratitis

– Gram-negative aerobic/facultative anaerobic bacilli:	– *Pseudomonas* – *Escherichia* – *Citrobacter* – *Klebsiella* – *Serratia* – *Proteus* – *Haemophilus*
– Gram-negative anaerobic bacilli:	– *Bacteroides* – *Fusobacterium*
– Gram-negative aerobic cocci and coccobacilli:	– *Neisseria* – *Moraxella* – *Acinetobacter*
– Gram-positive aerobic/facultative anaerobic cocci:	– *Micrococcus* – *Staphylococcus* – *Streptococcus* – *Aerococcus*
– Gram-positive aerobic bacilli:	– *Bacillus*
– Gram-positive anaerobic bacilli:	– *Clostridium*
– Actinomycetes and related organisms (aerobic):	– *Arachnia* – *Bifidobacterium* – *Mycobacterium* – *Nocardia* – *Streptomyces* – *Corynebacterium*
– Actinomycetes and related organisms (anaerobic):	– *Actinomyces* – *Propionibacterium*
– Gram-positive anaerobic cocci:	– *Peptostreptococcus*

dacryocystitis [11], has decreased in developed countries as a result of modern antibiotics and refinement in techniques for dacryocystorhinostomy [12]. *Staphylococcus* species continue to be the predominant cause of bacterial keratitis and in several reports *Staphylococcus epidermidis* or coagulase negative staphylococci (CONS) are the leading causes [13, 14]. *Pseudomonas* species is especially associated with daily or extended wear soft contact lenses related keratitis [15]. It is one of the commonest gram-negative bacteria causing microbial keratitis. Bacteria less frequently causing keratitis include *Corynebacterium* species [16], *Propionibacterium acnes* [17], *Bacillus* species [18, 19], *Neisseria gonorrhoeae*, *Listeria monocytogenes* [20], species of *Enterobacteriaceae* family [21], etc. *Nocardia* species are an important cause of keratitis [22]. Atypical mycobacteria such as *Mycobacterium fortuitum* [23], *Mycobacterium chelonae*, *Mycobacterium abscessus*, *Mycobacterium gordonae*, and *Mycobacterium avium-intracellulare* are infrequent causes of keratitis associated with corneal trauma or surgery. There are several reports of post excimer laser photorefractive keratectomy (PRK) and post excimer laser *in situ* keratomilieusis (LASIK) keratitis cases [24]. Caused by *Borrelia burgdorferi* a rare non-syphilitic spirochetal infection of the cornea has been reported [25]. Patients may

develop bacterial infection of the cornea post penetrating keratoplasty. Unlike conjunctivitis, fungi are a common cause of keratitis. A large variety of species have been reported (Table 15.2).

The commonest virus causing keratitis worldwide is herpes simplex virus (HSV). The HSV infection is usually a recurrent disease and the immunologic response associated with the episode. Sunlight, trauma (including surgery), heat, abnormal body temperature, menstruation, other infectious diseases, and emotional stress, etc., have been implicated in the activation of HSV infection. Recurrent epithelial keratitis occurs by reactivation of live virus leading to clinical manifestations such as dendritic and geographic ulcers. The dendritic or geographic ulcer may completely resolve leading to stromal scarring. One quarter of the patients may develop stromal disease, which may be infectious or immunological [26].

Table 15.2 Types of fungal species associated with fungal keratitis

– Hyaline septate filamentous fungi:	– *Fusarium species* – *Aspergillus* species – *Acremonium* species – *Beauveria* species – *Cylindrocarpon* species – *Geotrichum candidum* – *Neurospora* species – *Penicillium* species – *Paecilomyces* species – *Pseudallescheria boydii* – *Sphaeropsis subglobosa* – *Scopulariopsis* species – *Ustilago* species – *Volutella* species
– Dematiaceous septate filamentous fungi:	– *Alternaria* species – *Bipolaris* species – *Curvularia* species – *Cladosporium* species – *Drechslera* species – *Exserohilum* species – *Exophiala jeanselmei* – *Lasiodiplodia theobromae* – *Macrophomina phaseolina* – *Phialophora* species
– Yeasts and yeast-like fungi:	– *Candida* species – *Cryptococcus* species – *Rhodotorula* species – *Trichosporon* species
– Dimorphic fungi:	– *Blastomyces dermatitidis* – *Paracoccidoides brasiliensis* – *Sporothrix schenkii*
– Hyaline aseptate/sparsely septate filamentous fungi:	– *Rhizopus* – *Mucor* – *Pythium*[a]

[a]Parafungus

Acanthamoeba and Microsporidia are two keratitis-causing parasites, the latter being currently classified as parafungus. *Acanthamoeba* keratitis occurs in immunocompetent, healthy young individuals. While contact lens wear is the commonest risk factor for *Acanthamoeba* keratitis in the United States the common risk factor in patients of *Acanthamoeba* keratitis seen in developing countries is history of corneal trauma or exposure to contaminated water [27, 28].

Laboratory tests for diagnosis of conjunctival and corneal infections include direct microscopy, culture, antigen detection by direct or indirect fluorescent antibody (DFA, IFA) methods, conventional or real-time polymerase chain reaction (PCR), etc. Point-of-care tests are available for the detection of adenovirus and herpes simplex virus infections.

15.2 Role of Microbiology in the Diagnosis of Conjunctival and Corneal Infections

Diagnosis for any infectious condition begins in the clinics with the observation of clinical features that might be suggestive of a particular etiology. Microbiological investigations are aimed at either confirming or negating the clinical suspicion. They are indicated in situations where clinical features on their own may not be confirmatory. Excellent reviews have pointed out that most cases of conjunctivitis are self-limiting [29, 30] and obtaining clinical samples for microbiological investigations are reserved for neonatal conjunjuntivitis, recurrent conjunctivitis, conjunctivitis recalcitrant to therapy, severe purulent discharge, etc. The decision to procure clinical specimens for culture, antigen detection, or molecular tests is based on the likelihood of benefit to the patient. Interpretation of the tests requires understanding of the normal flora of the conjunctiva and the lids. In clinic rapid viral antigen testing is available for adenovirus and has a sensitivity of 86% and specificity of 94% [31]. This test can prevent unnecessary use of antibiotics limiting development of antibiotic resistance apart from saving considerable amount of money. An immunochromatographic assay kit for office-based herpes simplex virus detection has been mainly used in the diagnosis of HSV keratitis [32].

Generally, microbiological investigation of keratitis is indicated in sight-threatening (>1–2 mm) ulcers, in ulcers wherein an atypical organism is suspected, and in any ulcer that is not responding to therapy. However, in tropical and subtropical parts of the world where the prevalence of fungal keratitis is high, it is recommended to investigate all corneal ulcers. The methodology of sample collection and processing is described below.

15.3 Clinical Sample Collection

It is important to collect samples for the diagnosis of conjunctivitis from the conjunctival sac and for keratitis from the cornea. Tears or serum samples are rarely helpful in the diagnosis of corneal or conjunctival infections. Some of the crucial

guidelines include that the samples be collected carefully without contamination, without causing discomfort or pain to the patient and using appropriate technique in consultation with the microbiologist.

Some of the requirements for collection of samples include topical anesthetic eye drop (0.5% proparacaine hydrochloride), sterile cotton/dacron/calcium alginate swab, surgical blade number 15, Kimura spatula, glass slides (preferably cleaned with alcohol, hot air oven sterilized, wrapped in aluminum foil), and glass cover slips (preferably cleaned with alcohol). Culture media (liquid and solid), phosphate buffered saline pH 7.2, 95% ethyl alcohol and acetone for fixation, etc., are other requirements based on the intended investigations. Viral transport medium would be needed if viral culture is intended.

It is best to collect two conjunctival swabs if conjunctival discharge is present and send to the laboratory in sterile tubes. Moistened (with sterile saline or any liquid media) swabs may be used for optimum recovery of the organisms. While one swab could be used for making smears for direct microscopy, the other can be used for inoculation of culture media. Solid media could be inoculated initially followed by liquid media ending with breaking off the nonhandled distal end of the swab into the liquid medium. For viral culture, a dacron swab with conjunctival sample is agitated in cold viral transport medium. Wooden shaft of cotton or calcium alginate swab may inhibit viral growth. A conjunctival scraping under topical anesthesia using blade number 15 provides a good sample for direct microscopy from cases with minimal discharge with or without pseudomembrane.

The corneal sample is best collected using Kimura spatula or blade number 15 under topical anesthesia under slit lamp magnification in the clinic. Multiple scrapings are transferred on to glass slides and inoculated on culture media. If taken out of the refrigerator, the culture media should preferably be warmed to room temperature before use. The corneal scrapings are transferred on the solid media in multiple "C" shaped strokes. Slides and inoculated media are transferred to the laboratory in closed containers for immediate processing. An alternative method of collection of corneal sample using a single-sample device (Eswab, Copan Diagnostics, Inc) has been described that was comparable with the conventional method in the diagnosis of bacterial keratitis [33].

15.4 Sample Processing and Direct Smear Examination

In general, no transport medium (except for virus or *Chlamydia* culture) is recommended for conjunctival and corneal samples. Although McLeod et al. have described Amies transport medium without charcoal [34] to be effective transport medium, comparable to direct processing, in the investigation of fungal and bacterial keratitis, validation of this method by other investigators is lacking. Other useful samples in the investigation of microbial keratitis associated with contact lens wear would include contact lenses, contact lens cases, and contact lens solutions. These samples are directly processed for culture of bacteria, fungi, or parasites in appropriate media and smears are generally not made. Special procedures are required for

Table 15.3 Direct smear examination methods for the diagnosis of conjunctival and corneal infections

Type of sample	Type of organism/antigen to be detected	Staining methods for smears
Eye lash	• Parasites (Demodex, Lice)	• Saline/Glycerol Wet Mount
Conjunctival swabs/scrapings	• Bacteria, Fungi, Microsporidia	• Gram, Giemsa, KOH + Calcofluor White, Ziehl-Neelsen Stain
	• Viral antigens	• Direct/indirect immunofluorescence or immunoperoxidase assay
Corneal scrapings	• Bacteria, fungi, parasite (*Acanthamoeba*, Microsporidia)	• Gram, Giemsa, KOH + Calcofluor White, Lactophenol Cotton Blue, Gomori Methenamine Silver, Ziehl-Neelsen Stain
	• Viral antigens	• Direct/indirect immunofluorescence or immunoperoxidase assay

the collection of samples for the detection of parasites and viruses. Samples such as corneal buttons/biopsies need to be transported to the laboratory in sterile dry containers where they may be cut/minced into tiny pieces and inoculated on different culture media. Transport in normal saline is fraught with possibility of contamination. Direct smear examination methods that may be used for detection of various organisms from conjunctiva and cornea are shown in Table 15.3. Sample is transferred from the blade/spatula/swab to a glass slide over an area of approximately 1 cm circle marked on the reverse with a marker. While the specimen is thinly spread for dry smears (Gram, Giemsa, Gomori methenamine silver, etc.), it needs to be placed in the circle and covered with a coverslip without spreading, for wet smears (potassium hydroxide-KOH, calcofluor white-CFW, lactophenol cotton blue-LPCB, etc.). Preparation of at least two smears is recommended, which helps in confirmation of the findings. We prefer a combination of KOH + CFW, Gram, and Giemsa stained smears, which has provided us a high sensitivity and specificity for the detection of bacteria, fungi, microsporidia, and *Acanthamoeba* along with details of inflammatory cells in corneal scrapings. Fungi being uncommon in conjunctivitis, routine use of KOH + CFW is not justified for conjunctival samples. They are usually examined by Gram and Giemsa stains. A common binocular laboratory light microscope is adequate for the examination of the smears except when fluorescent stain such as calcofluor white is used which requires a fluorescence microscope. Ziehl-Neelsen stain using 20% H_2SO_4 or 1% H_2SO_4 (modified Ziehl-Neelsen stain or Kinyoun's stain) can be employed on the corneal scrapings when the Gram stain is suggestive of either mycobacteria (unstained, poorly stained bacilli in Gram stain) or *Nocardia* (thin, beaded, branching filaments). Gram stained smear can be decolorized and subjected to these special stains obviating the need to collect an additional scraping.

For polymerase chain reaction (PCR) the samples are placed in sterile phosphate buffered saline pH 7.2 and submitted to the laboratory where they may be retained at −20 °C until tested.

A rapid diagnosis of viral infection can be established by observing stained smears of corneal scrapings, conjunctival scrapings/swabs. Nonspecific staining techniques used for this purpose include Giemsa, Papanicolaou, and Hematoxylin-eosin stain [35]. Multinucleated giant cells, koilocytic changes, and intranuclear/intracytoplasmic inclusions, and types of inflammatory cells are some of the crucial information that can be obtained.

Conjunctival samples stained with Gram stain and KOH + CFW showing gram-positive cocci and microsporidial spores are shown in Fig. 15.1a–d. Corneal scrapings stained with Gram stain and Ziehl-Neelsen (ZN) stain showing gram-positive cocci, gram-positive bacilli, fungal filaments, budding yeast cells, *Pythium* filaments, *Acanthamoeba* cysts, microsporidia spores, etc., are shown in Fig. 15.2a–n. Figure 15.2m shows aseptate filaments of *Pythium* in the corneal scraping stained with Gomori methenamine silver (GMS) stain. Apart from fungal filaments, KOH + CFW is a highly sensitive stain for the observation of *Acanthamoeba* cysts, *Pythium*, yeast, and microsporidial spores (Fig. 15.3a–d and Fig. 15.1c). A gram stained smear can be decolorized with acetone and restained with either ZN or GMS

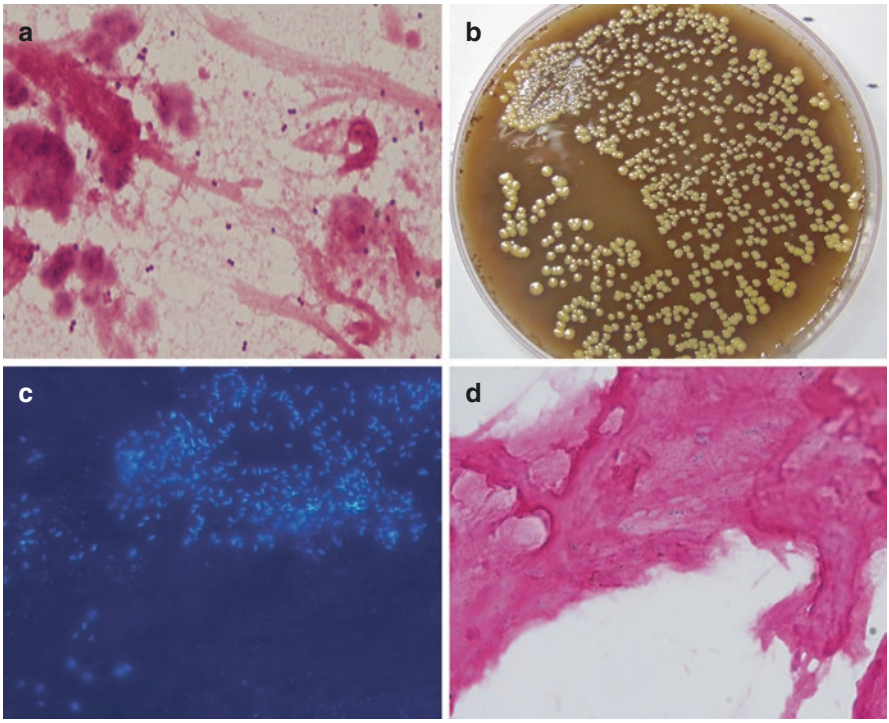

Fig. 15.1 (**a**) Conjunctival swab from a patient with purulent conjunctivitis showing spherical gram-positive cocci in singles, pairs, and in groups; (**b**) Confluent growth of golden yellow glistening colonies of *Staphylococcus aureus* on chocolate agar (37 °C, 24 h); (**c**) Conjunctival scraping showing microsporidial spores under fluorescence microscope (KOH + CFW, ×400); (**d**) Conjunctival scraping showing microsporidial spores with stippled gram-positive staining (Gram stain, ×1000)

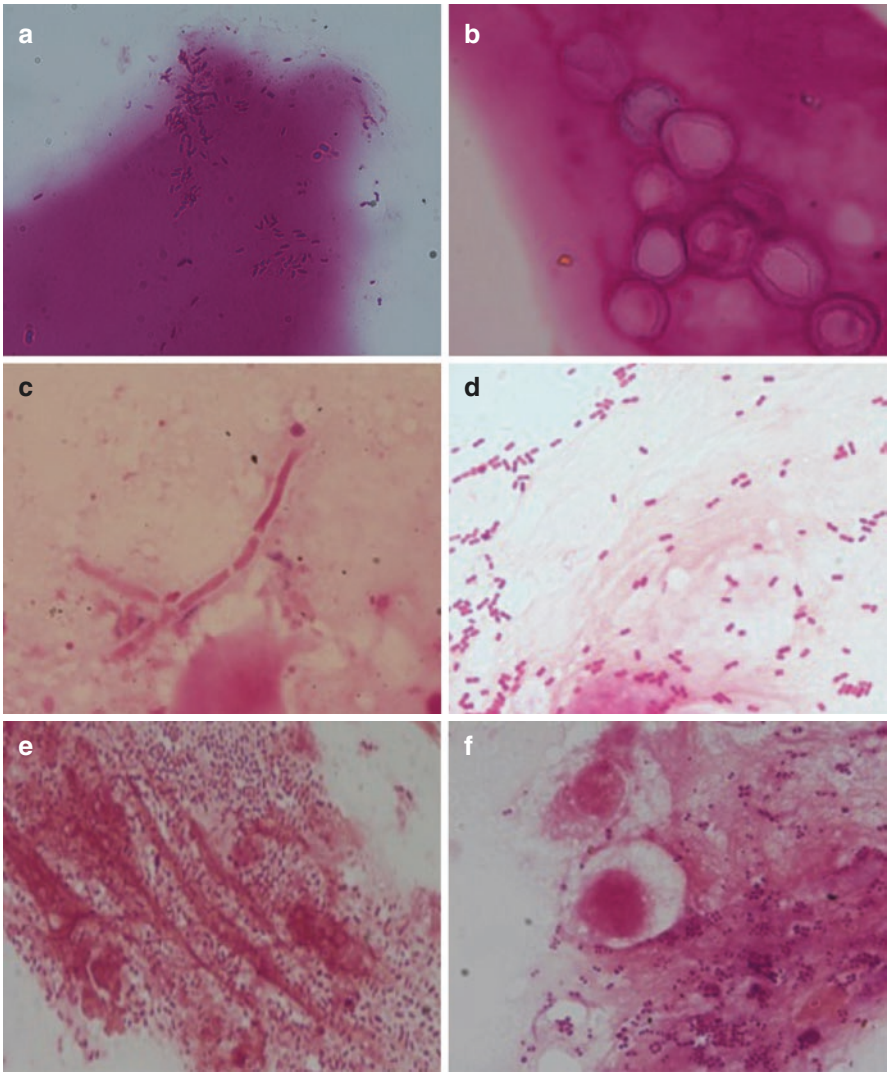

Fig. 15.2 Corneal scrapings showing: (**a**) Gram-positive beaded bacilli arranged in Chinese letter pattern suggestive of *Corynebacterium species* (Gram stain, ×1000); (**b**) Double walled *Acanthamoeba* cysts (Gram stain, ×1000); (**c**) septate, hyaline fungal filaments (Gram stain, ×1000); (**d**) Box-like gram-negative bacilli in pairs suggestive of *Moraxella species* (Gram stain, ×1000); (**e**) capsulated gram-positive cocci in pairs, suggestive of *Streptococcus pneumoniae* (Gram stain, ×1000); (**f**) Spherical gram-positive cocci in groups and short chains suggestive of *Staphylococcus* species (Gram stain, ×1000); (**g**) Microsporidial spores stained gram-positive (Gram stain, ×1000); (**h**) Budding yeast cells stained gram-positive (Gram stain, ×1000); (**i**) Gram-positive, thin, branching, beaded filaments suggestive of actinomycetes (Gram stain, ×1000); (**j**) Thin branching acid-fast filaments confirmatory of *Nocardia* species (Kinyoun stain, ×1000); (**k**) Poorly stained/unstained, beaded, gram-positive bacilli of *Mycobacterium species* (Gram stain, ×1000); (**l**) Slender acid-fast bacilli confirmatory of *Mycobacterium species*; (**m**) Aseptate, broad, branching filaments with ribbon-like folds suggestive of *Pythium species* (Gomori methenamine silver stain, ×400); (**n**) Broad, aseptate, branching filaments suggestive of *Pythium species* (Gram stain, ×1000)

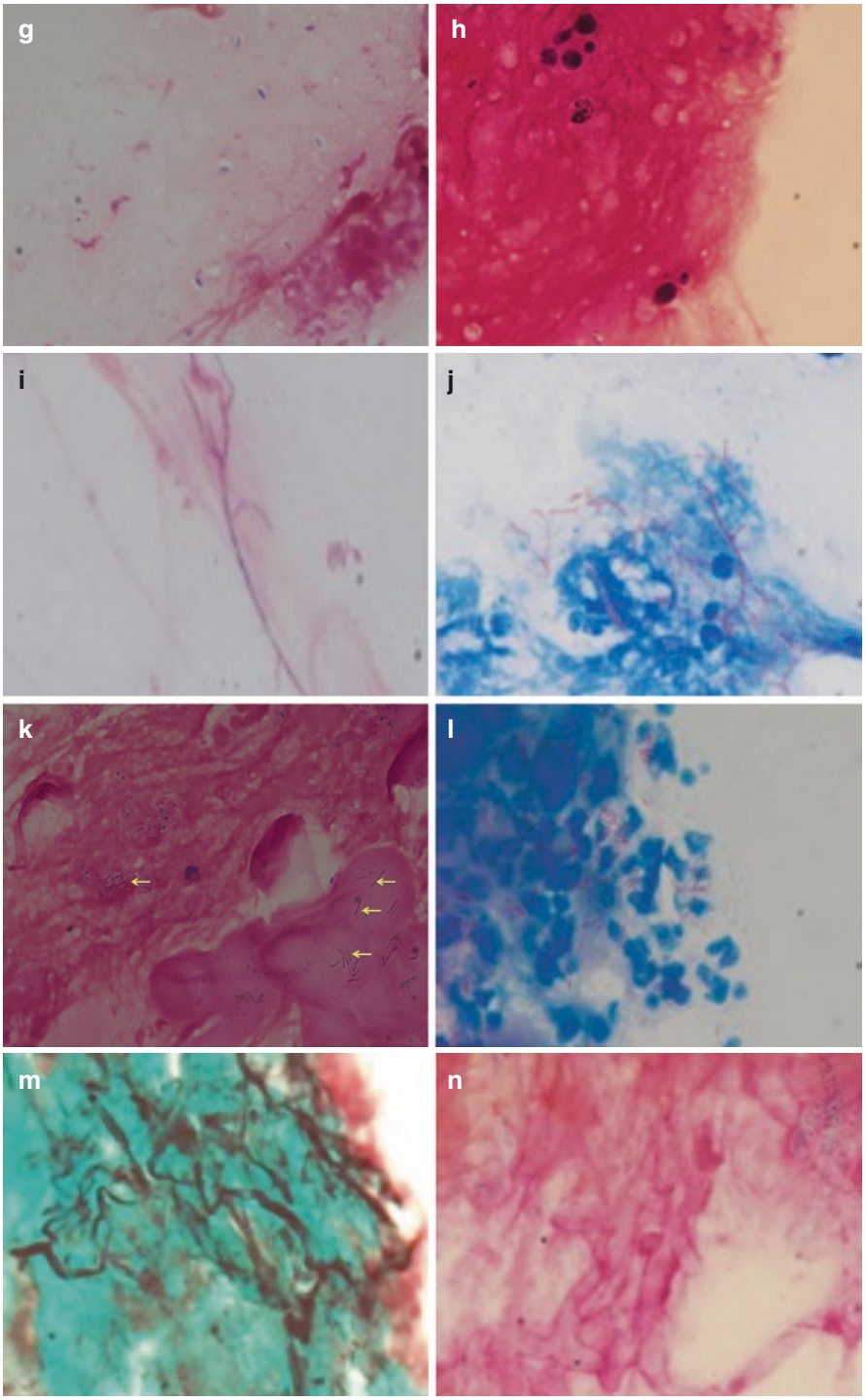

Fig. 15.2 (continued)

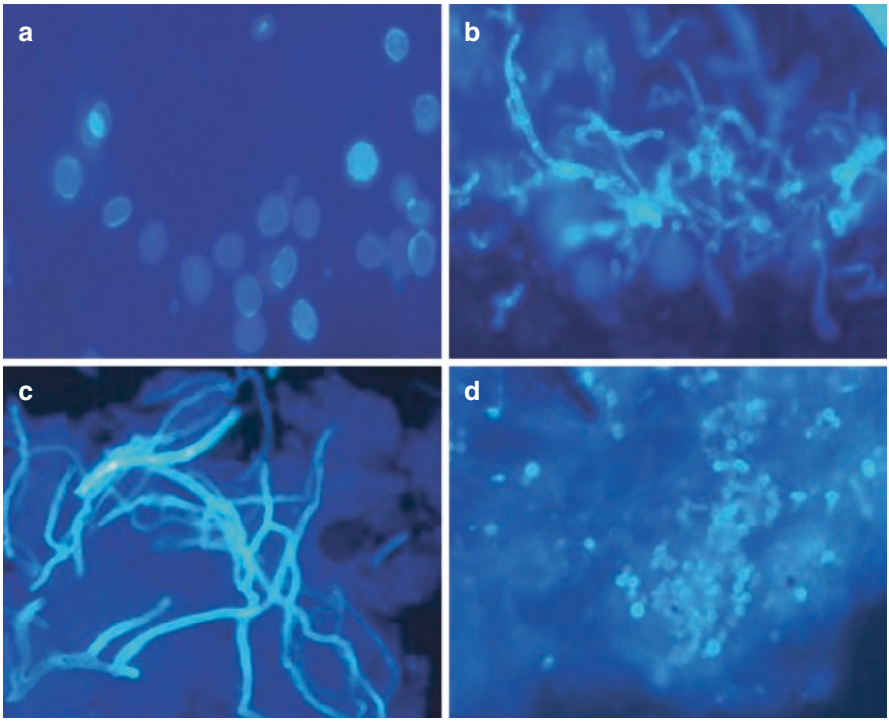

Fig. 15.3 Corneal scrapings stained with potassium hydroxide and calcofluor white (KOH + CFW) and observed under fluorescence microscope (total magnification ×400) showing: (**a**) *Acanthamoeba* cysts with characteristic hexagonal shape and double cyst wall; (**b**) septate, fungal filaments; (**c**) aseptate fungal filaments suggestive of *Pythium species*; and (**d**) round budding yeast cells

stain. Intranuclear inclusions are more efficiently seen in Papanicolaou stain than Giemsa stained smears; however, Giemsa stain is good for differentiating cell types. These staining techniques are rapid and inexpensive; however, they are often non-specific and have low sensitivity in the diagnosis of viral infections.

Specific cytology techniques used for viral diagnosis are techniques that indirectly suggest the presence of viral antigen in the clinical sample. Detection of cell associated viral antigen in a corneal scraping or conjunctival scraping is very useful in the diagnosis of viral infection. Corneal scraping should be made into a smear on glass slide and fixed in cold acetone (4 °C) prior to immunofluorescence assay. The commonly used assays constitute direct and indirect immunofluorescence and indirect immunoperoxidase assays for the diagnosis of HSV, VZV keratitis, and adenoviral keratoconjunctivitis. Figure 15.4 shows apple green fluorescence in the corneal cells positive for HSV 1 antigen by indirect immunofluorescence assay. These tests are rapid, specific, and sensitive, especially when suitable monoclonal or purified polyclonal antibodies are used. Relatively higher sensitivity and lower specificity is achieved with purified polyclonal antibody tests while monoclonal antibodies show high specificity but lower sensitivity. Indirect immunoperoxidase (IP) assay has

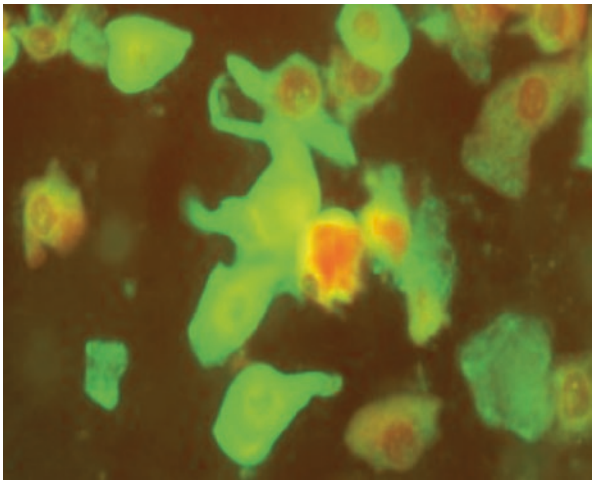

Fig. 15.4 Corneal scraping under fluorescence microscopy showing apple green fluorescence suggestive of presence of herpes simplex virus-1 antigen in the epithelial cells (indirect immunofluorescence assay). *Reagents*: Polyclonal rabbit antibody anti-herpes simplex virus type 1, and polyclonal swine anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (FITC)

Table 15.4 Culture media for the diagnosis of conjunctivitis/nonviral keratitis

Media	(1) Blood agar—aerobic
	(2) Chocolate agar
	(3) Brain heart infusion broth
	(4) Thioglycollate broth
	(5) Robertson's cooked meat broth
	(6) Non-nutrient agar
	(7) Sabouraud dextrose agar
Optional media	(1) Blood agar—anaerobic
	(2) Potato dextrose agar
	(3) Lowenstein-Jensen medium

distinct advantage over indirect immunofluorescence (IF) assay. The former provides a permanent preparation for records and utilizes an ordinary light microscope for observation while the latter has the inherent problem of quenching (fading) of fluorescence and requires an expensive fluorescence microscope. While the IF technique provides better results with frozen tissue sections IP technique can be done on paraffin embedded tissue sections.

15.5 Culture of Bacteria, Fungi, and Parasites (*Acanthamoeba*)

We recommend a common procedure for the culture of bacteria, fungi, and *Acanthamoeba* from corneal scrapings, for the clinical features of nonviral keratitis caused by these organisms could overlap. Different media that could be used for culture of common organisms from the corneal and conjunctival samples along with the incubation temperature and period required for incubation are given in Table 15.4. Culture of viruses requires cell lines and a separate protocol as shown in Table 15.5.

Table 15.5 Methods of transportation of specimens to the virology laboratory for investigation of viral conjunctivitis/keratitis

(A)	Corneal scrapings
	(1) Smear on glass slide, air dry, and send for staining/IF/IP
	(2) Transfer in a vial (0.5–1 ml) of viral transport medium (VTM) and send for culture. It can be stored at 4 °C without freezing
	(3) Transfer on a cellulose acetate membrane, air dry, fix in acetone/methanol, and send for staining/IF/IP
	(4) Transfer in 1 ml of PBS/MEM/HBSS and send for PCR
(B)	Corneal impression smear on glass slide or cellulose acetate membrane, air dry, fix in acetone/methanol/15 min, and send for staining/IF/IP
(C)	Corneal/conjunctival swab
	(1) Use dacron/cotton swab to collect material and transfer in VTM and send for culture. It can be stored at 4 °C without freezing
(D)	Corneal button
	(1) Place in VTM and send for culture
	(2) Place in 10% buffered formalin and send for histopathology
	(3) Place in PBS/MEM/HBSS and send for PCR

The number and type of media may be chosen as per the availability of the samples and clinical suspicion. However, we recommend use of media that would allow growth of bacteria, fungi, and *Acanthamoeba* in all clinically determined nonviral keratitis. For the investigation of conjunctivitis one could restrict to media for bacteria and fungi. All media are incubated aerobically at 37 °C except Sabouraud dextrose agar and potato dextrose agar that require 25–27 °C (BOD incubator). Chocolate agar is incubated in 3–5% CO_2 in a candle jar or CO_2 incubator. Owing to presence of oxygen reducing agents anaerobic organisms can be grown in thioglycollate broth and Robertson's cooked meat medium incubated aerobically in conventional bacteriological incubator. To grow anaerobes on blood agar it needs to be incubated in anaerobic chamber or anaerobic jar with gas pack.

All media are incubated for minimum of 1 week (can be extended to 2 weeks) and examined daily for growth. Bacteria such as *Nocardia* species, atypical mycobacteria, and *Acanthamoeba* grow slowly and require prolonged incubation. Although most fungi associated with eye infections are saprophytes and grow within a week, they may require incubation for 2–3 weeks for proper sporulation and identification. It is advisable to extend the incubation period for fungus or *Acanthamoeba* in presence of suggestive clinical features or detection of the organisms in direct microscopy.

Size, color, texture, consistency, and number of colonies on the inoculation marks on solid media are recorded. An approximate semiquantitative growth estimation may be recorded as one plus (<10 colonies), two plus (10–20 colonies), and three plus or confluent (>20 colonies) growth. While bacterial and fungal colonies are examined with unaided eyes, handheld magnifying lens may be used to observe small colonies. Observation of *Acanthamoeba* growth, on the other hand, requires use of light microscope. We prefer to place the non-nutrient agar plates (with lid on) under 4× or 10× objective lens of the microscope to avoid contamination due to repeated opening of plate. The refractile, irregular structures of trophozoites with acanthopodia appear in the vicinity of the corneal scraping inoculation mark on the

surface of the medium. One may be able to see the characteristic track marks made by the migration of the trophozoites on the *E.coli* lawn. Normally a trophozoite can be seen at the end of each track. With passing time the trophozoites are seen to convert into cysts. Unlike bacteria and fungi no colonies are formed by *Acanthamoeba* that are visible to unaided eye.

Production of turbidity indicates growth in liquid medium that need to be subcultured (transfer with loop on to a solid medium) and Gram stained for identification. Since it is common for culture media that are made in the laboratory to get contaminated, utmost quality control in the form of prior incubation and batch testing with known standard strains are required. In addition, it is helpful to follow certain guidelines to determine the significance of growth on culture media from conjunctival and corneal samples.

The growth of bacteria or fungus in culture is considered significant if the growth is more than 10 colonies (++) or confluent on the site of inoculation on solid media (Fig. 15.5b), or the organism was seen in direct microscopy (Fig. 15.5a), or if the same organism is grown in more than one medium.

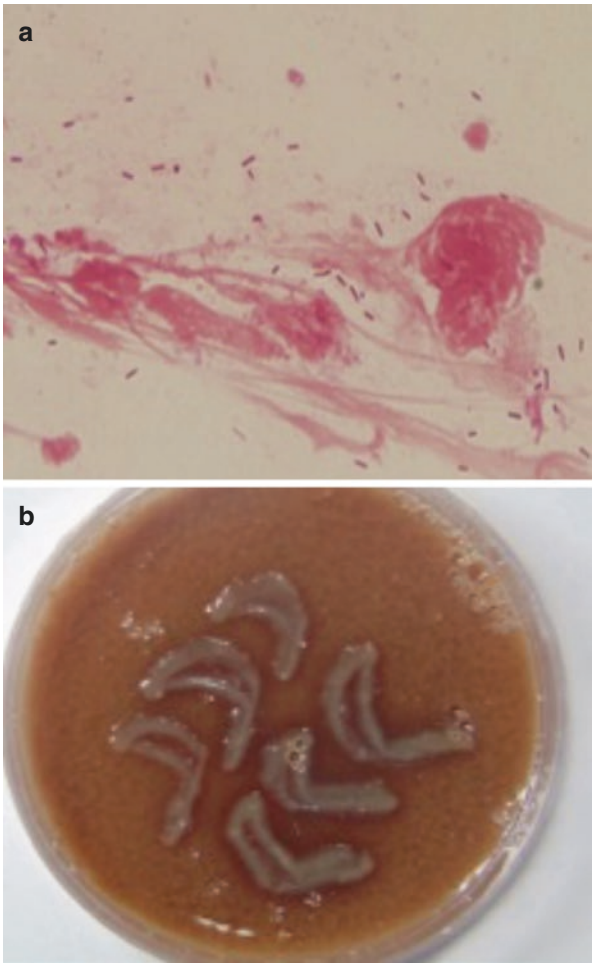

Fig. 15.5 (**a**) Gram stain of corneal scraping showing slender gram-negative bacilli suggestive of *Pseudomonas* species and (**b**) Confluent growth of *Pseudomonas aeruginosa* on chocolate agar (37 °C, 48 h)

15.6 Identification of Bacteria and Fungi

Standard microbiological procedures are adequate to establish the genus and species of the bacterial or fungal isolates from corneal and conjunctival samples. Most microbiology laboratories follow conventional biochemical tests. However, these are now being replaced with modern automated systems such as Vitek 2 compact system, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF-MS) for the identification of bacteria and yeast. Generally, a combination of conventional and automated methods may be needed. Filamentous fungi continue to be identified on the basis of colony characteristics and microscopic features. Several websites such as "doctor fungus," "mycology online," and Atlas of Clinical Fungi are helpful in morphological identification, apart from standard textbooks. Role of DNA sequencing of conserved genes in the identification of organisms is fast catching up in many laboratories and has become routine in some. Special tests may be required for identification of some species (zoospore formation by *Pythium insidiosum*) [36].

15.7 Culture of Viruses

Currently, isolation of viruses is not considered a preferred method for the diagnosis of viral infections, for the expense, expertise, and time that it entails. The sample for viral culture needs to be collected in an appropriate transport medium and sent to the laboratory while kept in ice pack. Methods of transport would vary according to the type of sample collected. Hank's balanced salt solution or 2 Sucrose Phosphate broth may be used [35].

Classically described techniques of virus isolation of embryonated eggs and animal inoculation are no more favored by most virology laboratories for routine diagnosis of viral infections. Immortal cell lines such as HeLa, Vero, HEp 2, MRC-5, and human corneal epithelial cell line have been used for isolation of herpes simplex virus from corneal scrapings [37].

Viral growth in cell lines is determined either by characteristic cellular changes, i.e., cytopathic effect (CPE) or by IF or IP techniques, which detect viral antigens in the infected cell lines. Initiation of CPE may take several days but antigens can be detected even before CPE occurs, thereby rendering the latter a more rapid method. For rapid detection of virus antigens in cultured cells the often used technique is that of shell vial technique. It is a modification of conventional tissue culture technique wherein entry of virus into the monolayer of susceptible cells (on a cover slip in a vial) is facilitated by centrifugation (spin amplification) of the vial containing cells and the clinical sample. The virus growth occurs in a shorter period (18–72 h) by this method and additionally, both IF and IP techniques can be performed easily on the cover slips retrieved from the vials for antigen detection.

Serologic tests for herpes simplex virus neutralizing antibody or complement-fixing immunoglobulins may show a rising antibody titer during primary viral

infection but have no value in recurrent infection. Since majority of the adult population is latently infected with herpes simplex virus, serological test is useful only when negative to rule out an infection.

15.8 Antibiotic Susceptibility Testing

In most laboratories the antimicrobial susceptibility testing of bacterial isolates is done by Kirby Bauer disc diffusion method, a qualitative assay, using CLSI (Clinical and Laboratory Standards Institute) guidelines [38]. CLSI (earlier known as NCCLS-National Committee for Clinical Laboratory Standards) is an US-based international, interdisciplinary, nonprofit, nongovernmental organization approved by FDA-USA and recommended by the world health organization (WHO). Bacterial isolates to be tested for susceptibility are made into a standard suspension and a lawn culture is made usually on Mueller Hinton agar over which commercially available antibiotic discs are placed followed by incubation. The drug diffuses radially in the medium producing a circular zone of inhibition if the organism is susceptible and smaller/no zone if resistant (Fig. 15.6b). Quality control includes standardization of the bacterial suspension, adequate thickness of the medium, appropriate incubation conditions, and accurate measurement of the zone of inhibition. The size of the zone of inhibition obtained is interpreted by referring to standard zone diameter interpretative chart that labels an organism as either susceptible, intermediate, or resistant to the antibiotics based on clinical breakpoints. The European equivalent organisation is EUCAST (European Committee for Antimicrobial Susceptibility Testing) which sets breakpoints for European Medicines Agency. Apart from clinical breakpoints for the interpretation of

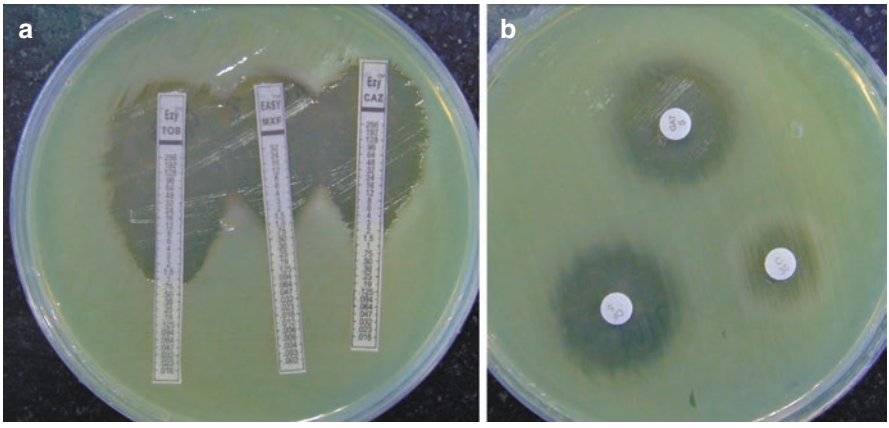

Fig. 15.6 Antibiotic susceptibility testing of *Pseudomonas aeruginosa* on Mueller Hinton agar by (**a**) E test showing elliptical zone of inhibition against the strip of antibiotic containing different concentrations of antibiotic along the strip and by (**b**) disc diffusion method showing zone of inhibition around the antibiotic discs

susceptibility tests it also provides epidemiological cutoffs for newer antibiotics. Although some differences exist between them both CLSI and EUCAST guidelines are acceptable across the world. Periodic attempts are made by the committees to harmonize the two systems. While the documents of CLSI guidelines come with a cost the EUCAST guidelines are free of cost on the website. Since the antibiotic disc content used in disc diffusion assays is based on serum level of the drug achievable by standard systemic therapy and does not represent the ocular levels of antibiotics, measurement of minimum inhibitory concentration (MIC) of the drug is considered a better parameter. Also, an infection caused by an organism resistant to an antibiotic by the test may respond to the drug for the reason that higher concentration of antibiotic can be delivered directly at the site of infection in the eye unlike systemic treatment.

MIC of a drug can be determined by several methods, Vitek 2 compact system being commonly used. Currently, many laboratories also use E test to determine MIC of drugs against bacteria and yeast [39, 40]. Earlier known as Epsilometer test, E test is a manual *in vitro* quantitative test to measure MIC of drugs with the ease of performing disc diffusion test. It employs a ready to use (commercially available from bioMerieux, France) plastic strip containing predefined gradient of an antibiotic. The strip is applied to the surface of an agar plate inoculated with the standard suspension of a test strain and the plate is incubated. The duration and temperature of incubation are predetermined based on the intrinsic growth characteristics of the organism. The MIC value is read from the scale in terms of μg/mL where the ellipse edge intersects the strip (Fig. 15.6a).

Certain drugs such as ceftazidime, vancomycin, piperacillin-tazobactam, colistin, and imipenem require MIC testing for confirmation of resistance indicated on the basis of Kirby Bauer disc diffusion assay. CLSI based broth microdilution method is used for testing susceptibility of filamentous fungi to antifungal drugs [41]. Although CLSI guidelines are available for testing MIC of yeast [42], aerobic actinomycetes (Mycobacteria, *Nocardia*) [43], and filamentous fungi [41], breakpoints for interpretation of the results are not available for all ocular fungi and actinomycetes. The testing methodology and guidelines are evolving concurrently with the increasing understanding of antifungal therapy for eye infections. Antifungal susceptibility testing is performed by very few laboratories and remains in the realm of research rather than routine owing to difficulty in testing and interpretation. Moreover, the panel of antifungal drugs available for therapy of ocular infections is too small to justify routine testing.

15.9 Molecular Methods

Microbiological tests for the diagnosis of infectious diseases have taken a leap in sensitivity and specificity by virtue of molecular techniques. These techniques basically detect the DNA of the organisms in clinical samples. They can also be used for detection of RNA. Presently they are the most sought after techniques for the detection of organisms that are difficult or expensive to culture or are unculturable. They

seem to be ideal for the diagnosis of viral infections for precisely these reasons. Apart from viral infections they have a big role to play in the diagnosis of corneal or conjunctival infections caused by Microsporidia, *Acanthamoeba*, *Propionibacterium acnes,* atypical mycobacteria, *Chlamydia trachomatis*, etc. Apart from detection of microbial DNA or RNA for diagnosis, these methods are extremely useful in the identification of organisms by DNA sequencing, a methodology that has opened up doors for recognition of heretofore unknown organisms. The commonly used methods include polymerase chain reaction (PCR) and real-time PCR that are briefly described below. Role of next generation sequencing (targeted or unbiased) of microbial DNA and RNA has added a greater dimension to the understanding of infectious diseases.

15.9.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is capable of amplifying DNA of organisms that may or may not be viable. Therefore, samples from patients treated with antibiotics can also be used for the detection of DNA of the organisms. Primers are designed to either help detect a particular organism or a group of organisms. Primers specific for a targeted organism have an overall limited value, however, are useful when clinical features point at specific organism such as *Mycobacterium tuberculosis*, herpes simplex virus, cytomegalovirus, and herpes zoster virus.

Panbacterial, universal, or eubacterial PCR targets conserved region of 16S rRNA gene and can be used for the diagnosis of bacterial keratitis. The detection limit by nested PCR can be as low as one organism. A multiplex PCR with gram-negative and gram-positive specific primers has also been described [44]. Several studies have reported application of panfungal PCR in the diagnosis of infectious keratitis. Primers based on 18S rRNA, ITS, or 28S rRNA genes have been used [45–47]. These PCR tests are reported to be highly specific and sensitive to the detection level of 1 fg of fungal DNA.

Generally, a second step is required to identify the species of bacteria or fungus following panbacterial and panfungal PCRs. Methods such as sequencing of the amplified DNA or hybridization with specific probes can be used. Sequence of the amplified DNA is compared with similar sequences in databases using BLASTn program of the National Centre for Biotechnology Information (NCBI, GenBank database). A score of 97% and similarity of 98% allows the genus recognition and a score of 99% or more may assign a species name. Phylogenetic analysis further confirms the species in relation to known sequences available in the database. It allows comparison with type strains available.

Hybridization technique constitutes transfer of amplified DNA on a membrane (dot blot assay), to which are added labeled (radioactive isotopes, fluorophores, haptens like biotin or digoxigenin or an enzyme) specific probes. The probes are usually a short strand of oligonucleotide specific for hybridization with complementary sequence of either one or a group of organisms. In DNA chip technology, complementary sequences to signature genes of specific organisms or groups of

organisms are dotted on the chip, to which is added multiplex PCR amplified biotin labeled denatured DNA (of the sample) that would hybridize to complementary DNA on the chip. In the next step enzyme labeled streptavidin would reveal the specific gene by color development on addition of the substrate [48].

Bacterial and fungal genome can be differentiated by their genetic fingerprint produced by use of restriction enzymes on the amplicons using PCR based Restriction Fragment Length Polymorphism (PCR-RFLP). The number of fragments is proportionate to the number of restriction sites in the genome and is specific for particular species [49]. With the availability of commercial agencies that help in sequencing at a reasonable cost, DNA sequencing is preferred over RFLP and hybridization techniques by most microbiologists. DNA sequencing and phylogenetic analysis allows recognition of unknown organisms [36].

In recent times, next generation sequencing (NGS) is evolving as a technology for precise identification of organisms. Unbiased metagenomic deep RNA sequencing has been applied to identify pathogens causing conjunctivitis [50].

Application of real-time qualitative or quantitative PCR in the diagnosis of infectious keratitis is increasing by the day. Quantitative PCR (qPCR) determines the amount of absolute or relative DNA in the sample. Real-time PCR requires a special thermocycler that measures fluorescence, which is produced in proportion to the amplification of the DNA, cycle by cycle. Commercial kits (herpes simplex virus, herpes zoster virus, cytomegalovirus, *Mycobacterium tuberculosis,* etc.) are available for diagnostic application of this technique. Being quantitative this test has a potential prognostic value to determine therapeutic response. Automated real-time PCR has been employed for rapid diagnosis of adenoviral keratoconjunctivitis [51]. Assessment of prevalence and clinical outcome of HSV type-1 [52], and quantitation in tear fluid and aqueous humor has been done using real-time PCR [53]. Several studies have shown the wide application of this test in the understanding of HSV keratitis such as detection of large number of HSV DNA copies in herpetic epithelial keratitis compared to stromal keratitis [54], and detection of HSV DNA in tears and saliva of 98% of tested asymptomatic individuals [55]. This technique was useful to demonstrate that Cytomegalovirus (CMV) is an important cause of corneal edema (endotheleitis) [56] and is the method of choice for the diagnosis of CMV endotheleitis [56].

Molecular techniques in the hands of microbiologists are evolving rapidly and are majorly impacting the understanding of infectious diseases of the eye. NGS of ocular samples has not only shown that healthy conjunctiva harbors many more genera of fungi than can be deciphered using culture techniques, but it has also unraveled dysbiosis in the fungal microbiome of conjunctiva and cornea in patients with fungal keratitis [57, 58]. Studies from L V Prasad Eye Institute, India have reported alterations in bacterial and fungal gut microbiome in patients with bacterial and fungal keratitis using NGS technology with far-reaching implications of possible role of probiotics in the control of these infections [59, 60]. More work in this direction is obviously warranted to understand the significance of the findings.

15.10 Conclusion

Accurate diagnosis of infectious diseases of the conjunctiva and cornea requires a well-equipped microbiology laboratory and well-trained staff. While conventional methods of microscopy and culture of clinical samples remain the gold standard enormous progress has been made with application of molecular methods in the diagnosis, which should be utilized when feasible along with appropriate interpretations that are clinically relevant.

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