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Laboratory in the Diagnosis of Bacterial and Fungal Keratitis

Virginia Vanzzini Zago and
Ana Lilia Perez-Balbuena

Additional information is available at the end of the chapter

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1. Introduction

Bacterial and fungal corneal infections are characterized by the presence of a replicating microorganism as the cause of inflammation, and loss of corneal epithelial cells and ulcers, as the last expression of inflammatory phenomenon [1]. Clinically, it is difficult to establish a diagnosis of bacterial keratitis specifically the causal agent. For this reason, the use of laboratory techniques is extremely important to establish a diagnosis. Clinical data created a suspected diagnosis, but results from the laboratory, particularly crops, studies represent the fundamental basis for a definitive diagnosis. Microbiological culture is the only way to determine the sensitivity to antibiotics and guide therapy to achieve optimal management of these conditions [2].

The active replication agents of the infectious ulcers can be viruses, bacteria, fungus *Acanthamoeba* or *Microsporidium*. An early detection of causative of microorganism helps to a better and specific medical treatment in order to reach a better prognosis, visual acuity and even preservation of the ocular integrity.

2. Natural history of a corneal infection

Colonization: Colonization is the first step after the arrival of pathogenic bacteria to corneal surface. The ability of bacteria or fungi for its adhesion to corneal epithelial cells defines its pathogenesis. After the adhesion phase, fungus or bacteria start an active replication supported by nutritional and temperature conditions of the tissue, reaching a bunch of living microorganism over the corneal epithelial cells.

Invasion: By means of proteases, lipopolysaccharides, streptolysins, dermonecrotic staphylococci, the microorganism can breakdown the epithelial layer cells and originate a corneal epithelial ulcer or crossing Bowman layer, in some cases reaching corneal stroma. This tissue invasion can be observed in the slit lamp and it is described as desepithelization due to the loss of surface epithelial cells.

Multiplication: In the surface of epithelial layers, or because of a traumatism some microorganisms reach corneal stroma, finding good conditions in nutrients and temperature for an active multiplication and liberation of harmful substances that initiate the inflammatory phenomenon.

Inflammatory response: As a response of the invasion of pathogenic microorganism, the corneal tissue elaborates some potent mediators substances for inflammatory and immune response named cytokines, synthesized mainly by lymphocyte cells, chemotactic, and tumor necrotic factors (TNF). The first sign of inflammatory response is edema by accumulation of interstitial water between epithelial cells itself and keratocytes.

Migration of leukocytes. By diapedesis phenomenon, the migrating leukocytes arrive to infiltrated corneal tissue, from new vessels formed on clear cornea or from limbus, after this fibrin and collagen IV accumulation into deep corneal stroma form an evident infiltrate. The role of polymorphonuclear leukocytes part of innate immune defense, mainly is based on his ability of ingest bacteria and digest it, by the oxygen dependent killing pathway or by potent oxidants like hydrogen-peroxide, hydroxyl radicals, chloramines and hypochlorous acid. In fungal keratitis, extensive migration of polymorphonuclear neutrophils (PN), around fungal hyphae in order to destroy it, plasma cell and in some cases eosinophils are observed. The dead of inflammatory cells (PN) contribute to the destruction of surrounding corneal tissue because the release of lysosomal enzymes and oxygen metabolites.

Anterior chamber inflammatory reaction: The arrival of leukocytes and fibrin to anterior chamber is called flare and the accumulation of inflammatory cells (PN) is visualized like hypopyon, this phenomenon can be accompanied by inflammation of the endothelial tissue with fibrin small spots named retrokeratic deposit.

Scar: The last step of an infectious keratitis is the accumulation of fibrin in the site of corneal wound or where invasive infectious process has begun, and form a permanent scar that, depending on its size and localization, can permanently low the visual acuity. [3]

3. Corneal predisposing factors in corneal infections

- a. **Local corneal and systemic factor.** The risk factors that predispose to corneal infection involve a breakdown of normal defense mechanism like in diabetes, Sjögren or any kind of systemic immunosuppressant that helps the invasion of pathogenic microorganism.
- b. **Corneal trauma.** It is one of the most frequent predisposing factors for bacterial and fungal keratitis. Ocular surface disorders like, dysfunctional tear film and dry eye syn-

drome, epithelial abrasion with mineral or organic foreign body, trauma due to surgeries, long lasting use of contaminated contact lens and toxic agent are considered as local risk factor for both corneal infections, and with the presence of one or more of these events, even local conjunctiva microbioma as coagulase negative *Staphylococcus* are capable of causing corneal inflammatory response.

- c. **Erosions and dry eye.** Preexisting ocular disorders located in the eye lids as *Staphylococcus aureus* blepharitis or apposition of eyelashes can cause erosions in corneal epithelium. Corneal exposures for old scars in the palpebral border may affect the perfect ocular occlusion and may be the cause of corneal epithelial desiccation and micro-erosions. Tear film dysfunction and dry eye syndrome after the 5th decade of life are the main cause of epithelial irregularities in corneal surface in males and females.
- d. **Corneal wounds and scars.** The corneal surgeries like penetrating or lamellar keratoplasty, kerato-refractive (Laser In Situ Keratomileusis, Photo Refractive Keratoplasty or any other surgery), corneal sutures or limbus wounds may open the corneal tissue to microbial entry of bacteria or fungus to epithelium or stroma, the subsequent microbial proliferation and tissue inflammation leads to the infectious keratitis.

4. Bacterial pathogenesis

- a. **Adherence:** Bacterial adherence occurs when the pathogenic or non pathogenic bacteria adheres over the corneal wounded or normal epithelial cells, using their bacterial adhesin for attachment and beginning the colonization on the glicocalix; one glycoprotein located over the epithelial corneal cells. After this step the bacterial multiplication and subsequent invasion can cause an inflammatory response and severe ulceration as is observed in corneal keratitis caused mainly by pathogenic species of *Listeria monocytogenes*, *Streptococcus pneumoniae* and *Neisseria gonorrhoeae*.
- b. **Evasion of corneal defense.** Some indigenous bacteria of the conjunctiva, like coagulase negative *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* lives in conjunctiva attached by means of adhesines evading the antibodies and multiple defense substances solute in ocular tears film and ocular blinking because the conjunctiva is his own habitat [4].
- c. **Toxins and proteases.** Bacterial exotoxin and endotoxin can cause severe inflammation like the endotoxin in *Pseudomonas aeruginosa*, *Serratiamarcescens*, *Enterobacter cloacae* and exotoxin staphylolisin of *Staphylococcus aureus*.
- d. *Pseudomonas aeruginosa* produce an exotoxin A, capable of causing inhibition in protein synthesis in mammalian cells by the same mechanism as diphtheria toxin. It also produces two extracellular proteases (elastase and alkaline protease) that have tissue-damaging activity capable of degrading complement and coagulation factors [5]. Experimentally in corneas of mice and rabbits, it was described a novel *Pseudomonas* protease IV (exoprotein) that contribute to corneal surface and stroma virulence [6].

- e. **Multiplication capability.** *Streptococcus pneumoniae* shows an accelerated multiplication rate and leukocytes chemotactic factors synthesis that may originate a huge inflammatory cell accumulation in anterior chamber. *Enterobacteriaceae* like *Serratia*, *Enterobacter*, *Escherichia* etc. have a multiplication rate of two hours for each generation cell, and its growth is exponential. Depending on the adaptability and the capability of the invading microorganism, like quick reproduction and production of toxic substances as results of his own metabolism, both factors known as virulence, the keratitis caused by the bacterium mentioned above rapidly progress to corneal edema, epithelial ulcer, and dense infiltrate. [7]
- f. **Bacterial corneal invasion.** The bacterial invasion to corneal epithelium or corneal stroma is the first step to a severe inflammatory process, with the attraction of inflammatory substances and the initiation of edema and disruption of cellular union on the corneal epithelial cells. Yi [8] demonstrated that Gram negative bacteria lipopolysaccharide attaches on Occludin ZO-1 and ZO-2 disrupting the tight cellular junction in rat corneal epithelium and human cultured cells.

5. Fungal pathogenesis

- a. **Fungal adhesive mannoproteins.** The mannoproteins regulate the attachment of yeast and filamentous fungi to corneal epithelial cells. The cell wall of *Candida albicans* is composed mainly by the polysaccharides mannan, glycan and chitin, in this yeast the ligands proteins for the attachment cell are mannoproteins too. The presence of large quantities of mannoprotein reveals its pathogenic capability.

The outer fibrillar layer in filamentous fungi and yeast is composed by mannoprotein described as an external coat, which regulates the attachment; this coat is sloughed off during the invasive phase in a corneal infection.

In *Aspergillus fumigatus* keratitis have been demonstrated toll-like2 and 4 (TLR2, TLR4) cell receptors as participants in inflammatory response as key of innate immune system that triggers host defensive responses inducing interleukins IL-1 β and IL-6. [9]

- b. **Fungal corneal penetration.** Fungal corneal infections always begin in the surface where traumatic events happen, then the fungal cells grow in the surface because the fungi are mainly aerobic microorganism; however, pathogenic *Candida* can reach deep corneal stroma in his hyphae cell form. In some cases, contaminated trauma involve deep cornea, the fungal cells reach the corneal surface after, in both cases the arrival of polymorphonuclear leukocytes and fibrin make a dense infiltrate after the third week of the inflicted cornea.
- c. **Fungal toxins.** The investigations about the harmful action of fungal toxins or micotoxines in corneal infections are beginning nowadays. In keratitis due to *Aspergillus flavus* have been demonstrated aflatoxin B1 in 80% of strains isolated from human corneal infection, and only in 40 % of *Aspergillus flavus* strains isolated from the environment. [10]

6. Corneal innate defense

- a. **Epithelial integrity.** The intact cornea epithelium cells act as an effective barrier that avoids the entrance of bacteria and fungi. Trauma and hypoxia caused by contact lenses or dry eye may cause corneal epithelial cells loss opening the sites for a bacterial or fungal infection.
- b. **Limbo stem cells.** The constant renovation of corneal cells, moving across the epithelium surface to the corneal center, is a renewal system of cells on the outer layer making new and strong unions between them; this renewal mechanism is also important for the wound reconstruction.
- c. **Phagocyte phenomenon.** It is a non specific, innate immune system form for clear bacteria, and it is very important in acute bacterial infections (Figure 1) diminishing bacterial multiplication by digesting it by several ways as mentioned before.
- d. **Tear layer.** This important aqueous-lipidic layer is a rich water soluble components barrier, it have immunoprotein mainly secretory IgA, immunoglobulin G, complement, lysozyme and ferritin that affect bacterial cell wall.

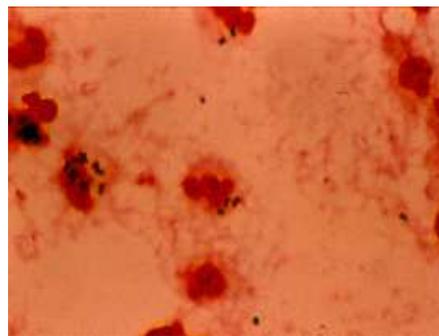


Figure 1. *Streptococcus pneumoniae* fagocitized in the protoplasm of Polymorphonuclear leucocyte in Gram stained smear of corneal ulcer. 1000 X magnification.

7. Problem statement

Viral keratitis is more often diagnosed in any ages in developed countries. Bacterial keratitis related to contact lens use and subsequent to a conjunctivitis are more frequent than fungal corneal ulceration often caused by trauma contaminated with organic soil in patients with agricultural or related activities, in developing and undeveloped countries.

All this corneal infections are important cause of visual loss in children or in males and females in productive ages; for these reasons, it is very important an early clinical and microbiological diagnoses and specific medical or surgical treatment in order to reach a better visual prognosis for those affected patients.

8. Application area

The area of application of this descriptive study, is in the diagnosis of corneal infections, and specifically related to the support of the laboratory in the diagnosis of bacterial and fungal keratitis.

9. Laboratory methods: The support of the diagnosis in bacterial and fungal keratitis

In all inflammatory corneal infection it is recommended in the first consultation, for diagnostic purposes, to take a small sample of corneal secretion of the surrounding or central zone of the ulcer, taking care of avoiding being so invasive. In imminent perforation risk keratitis, the best site is in the edges of the ulcer, and it is the best to take the sample with cotton swab, alginate [11] or Kimura spatula for seeding the sample in the cultures mediums.

Before taking the corneal sample, 2 drops of topical anesthetic (tetracaine 5 mgs/ml) are applied before obtaining smears from the corneal ulcer with heat sterilized and cold Kimura spatula, or with the sterile cotton or alginate swab as mentioned before, in order to be seeded in Petri dishes with culture medium in C streaks, in a wide variety of medium; blood agar (5% sheep blood in brain heart agar base), chocolate agar (1% enrichment supplemented) incubated in 4-5% CO₂ ambient at 37° centigrade for bacterial growth. For fungus cultures, Biggy agar slant for *Candida*; Sabouraud dextrose 2%, or Sabouraud Emmons both with 0.01% cloramphenicol and without cycloheximide inhibitor agar slant, with incubation at 27° centigrade and daily observation for a minimum of 3 weeks [12].

In the same way, three samples it have to be taken for making three smears in the center of each previously cleaned slide marked with a circle made with glass pencil for staining and microscopic observation.

Microscopic examination to search bacteria and fungi was made in each case by periodic-acid Schiff (PAS), Giemsa and Gram or in some cases by Zihel-Neelsen for acid-fast bacilli according to Prophet [13], and in few cases with calcofluor-Evans Blue (Cellfluor) and epifluorescent light microscopy. (Figure 2)

In keratitis cases diagnosed with concomitant anterior or posterior endophtalmitis, the samples of aqueous and vitreous humors, can be taken in the surgical room by an ophthalmologist and sent to the laboratory in the same syringe, in which the samples were collected. These samples will be seeded in the cultures mediums as described before and in conventional mediums for anaerobic incubation. The isolated bacteria can be identified by conventional, automated or semi automated test.

The fungal yeast isolated from corneal scrap samples may be identified by AUXACOLOR® 2 (Biorad® France) absorption sugars kit for *Candida*, cell germination forming pseudomycelium (Figure 3) and microcultures in corn meal agar with cover glass over the seed, by the characteristic organization of hyphae, and chlamydospores in each *Candida* specie.



Figure 2. Calcofluor-Evans Blue stain and florescence microscopic view of a corneal smear in a keratomycose in a case patient



Figure 3. *Candida albicans* pseudomicelium test stained with PAS 1000 X

Recommendations	
1. The sample must be taken before the start of treatment	
2. It is needed the use of 2 drops of topical anesthetic (tetracaine 5 mgs/ml) before scraping any corneal lesion.	
Giemsa Cytology	
Polymorphonuclear leukocytes +++ Bacterial or fungal keratitis	
Polymorphonuclear leukocytes +++, Eosinophils ++ Allergic keratoconjunctivitis	
Lymphocytes and macrophages +++ Viral or toxic keratitis.	
Mico-organism	Stain
Bacteria and fungus	Gram
Fungus	Periodic-Acid Schiff, calcofluor-Evans Blue
Mycobacteria	Ziehl-Neelsen
Actinomycetes (Actinomyces, Nocardia)	Kinyoun
Parasites (Acanthamoeba)	Giemsa, calcofluor-Evans Blue (Cist).

Table 1. Clinical and laboratory features in bacterial and fungal keratitis

For white filamentous and melanized fungus cultures, it may be observed for its morphology and pigmentation, on surface and reverse of the colony and for its final identification in microcultures for his characteristics conidial forms, prepared with lactophenol blue and direct optical microscopy observation according to the Manual of Clinical Microbiology [14] and Larone [15].

10. Gram positive bacteria and *Mycobacterium*

Coagulase negative *Staphylococcus*. Frequently keratitis associated to coagulase negative *Staphylococcus* are located in the paracentral sites or even near the limbo in patients whom have some systemic immunologic involvement like arthritis or Sjögren syndrome. The main species isolated are *S hominis*, *S haemolyticus* and in all cases the bacteria came from conjunctiva flora. In all series consulted *Staphylococcus epidermidis* are the most frequent keratitis bacteria isolated [16].

***Staphylococcus aureus*.** Keratitis caused by this bacterium begin as an epithelial defect, in concomitant conjunctivitis, indiabetic patients or treated with topical steroids patients, beginning as a superficial and stroma, multifocal opacity with few inflammatory cells. After two or three days without antibiotic treatment, a dense infiltrate is observed in the immediate area below the ulcer, and it may progress in indolent form or take a rapid development with a deep and abundant secretion over the corneal surface and conjunctiva. At same time, it can be observed an important ciliary inflammatory reaction close to the ulcer, and conjunctiva vascularization,(Figure 4). In some patients, a sterile ulcer located in the inferior corneal zone (8 to 4 clockaround) are caused by immune reaction to dermonecrotic toxins and staphylolysin generated by *Staphylococcus aureus* blepharitis demonstrated by cultures of both; eye lids superior and inferior (Figure 5).

The corneal smears showed inflammatory cells and Gram positive intracellular or extra-cellular round bacteria (Figure 6). In a diabetic patient, the sample cultures yielded abundant colonies of *Staphylococcus aureus* in cornea and in conjunctiva samples (Figure 7).

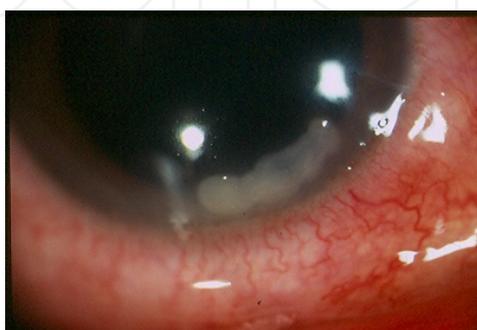


Figure 4. *Staphylococcus aureus* near the limbus keratitis and ciliar reaction.



Figure 5. Immune inflammatory reactions in cornea, due to *Staphylococcus aureus* toxins in a patient diagnosed with bacterial blepharitis.

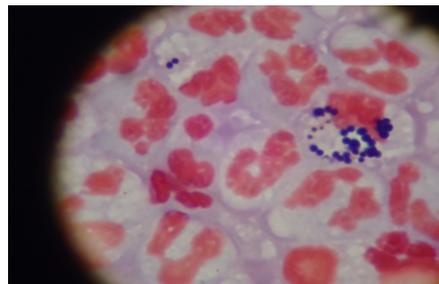


Figure 6. *Staphylococcus* cells ingested by polymorphonuclear leukocytes in a Gram smear of bacterial keratitis. 1000 X



Figure 7. Culture of *Staphylococcus aureus* keratitis showing incorneal C streaks and conjunctiva sample abundant colonies, from a diabetic patient

Streptococcus pneumoniae, S viridians and S agalactiae. Formerly named serpinginous ulcer, begins in central cornea with a focal suppurative stromal infiltrate that can reach superficial spread with leading edges, and dense infiltrate below the ulcer (Figure 8), in 70% of the case, hypopyon is observed in some cases occupying 50 % or more in anterior chamber and abundant conjunctiva yellowish secretion, patients refers severe pain. In diabetic patient and treated with topical steroid, the severe inflammatory process that reaches vitreous can cause an inflammatory or infectious endophthalmitis.

In infrequent contaminated post LASIK surgeries, keratitis is originated on streptococcal conjunctivitis developed after the surgery and it is observed like white inflammatory spots in the inter-phase wound and below the corneal flap. (Figure 9)



Figure 8. Central, suppurative *Streptococcus pneumoniae* keratitis in a immuno suppressed female patient.

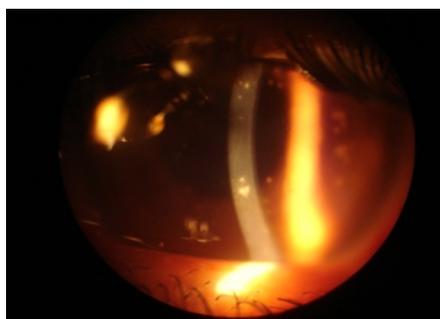


Figure 9. Post LASIK *Streptococcus pneumoniae* keratitis.

In the corneal samples smears, Gram positive *diplococcus* are observed. (Figure 1), and in cultures, *Streptococcus pneumoniae* colonies are obtained(Figure 10), the presumptive identification test for inhibition growth with cooper compound Optoquine is shown in Figure 11



Figure 10. Abundant colonies of *Streptococcus pneumoniae* in conjunctiva and cornea samples from patient of figure 9 (Left).

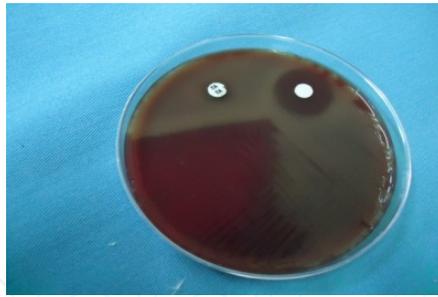


Figure 11. Optoquine inhibition test for *Streptococcus pneumoniae*, and Bacitracine disk without inhibition (Right).

Actinomycetes. Keratitis caused by anaerobic Actinomycetes like *Actinomyces israeli*, *A. bovis* or aerobic Actinomycetes classified in the genus *Nocardia*, *Actinomadura*, *Gordonia*, *Nocardiosis*, *Oerskovia*, *Rhodococcus*, *Streptomyces*, *Sacharomonospora*, *Thermmoactinomyces*, *Tsukamurella* [17], are indolent and with torpid evolution, without response to topical antibiotic like 4a generation quinolone, the traumatic and soil contaminated antecedent are considered in 30%, the infiltrate shows some dense spots in clear cornea and the corneal ulcer is above the infiltrate zone (Figure 12), the smears reveal filamentous Gram positive bacteria (Figure 13) and the culture show yellowish-white colonies in the C strakes of the corneal sample (Figure 14) the medical topical treatment recommended are topical amikacin or sulfadiazine and oral sulfamethoxazole and trimetoprim in regular doses. [18]



Figure 12. Keratitis due to *Nocardia* in a traumatic soil contaminated, 5 days of evolution in an adolescent male.

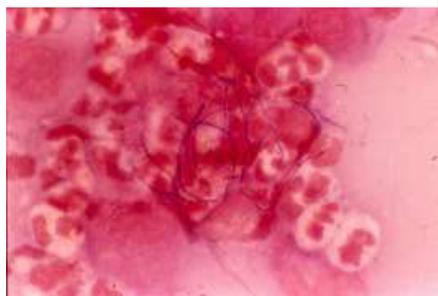


Figure 13. Gram positive filamentous bacteria showing ramifications in the cornea sample (Left).

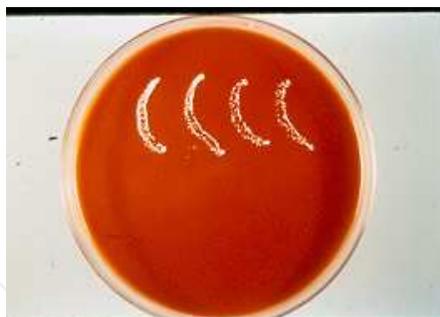


Figure 14. *Nocardia asteroides* cultures from cornea sample of patient in figure 12.

Non tuberculous Mycobacterium: *Mycobacterium chelonae*, *M intracelulare* are rapidly growing bacteria (7 to 8 days) and are related to low pain or indolent keratitis with torpid evolution, difficulty in diagnosis and with poor results in the treatment. The risky antecedent are corneal erosions or cornea transplant (Figure 15). The topical treatment needs long time and various antibiotics; fourth generation quinolones, amikacyn and clarytromycin. In the smears often it is observed Gram irregular and curved bacilli that in Zihel-Neelsen stain appear in a reddish color (Figure 16), and the cultures grows visible colonies in 7 days. (Figure 17)

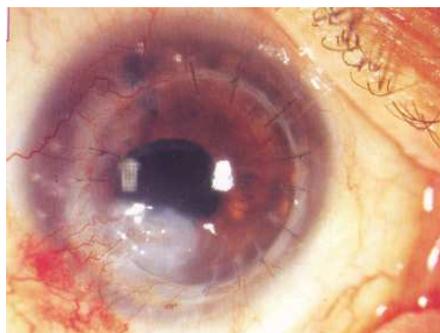


Figure 15. Post-penetrating keratoplasty contaminated with *Mycobacterium chelonae*



Figure 16. Corneal smear Zihel-Neelsen stained, showing acid-fast bacilli (black arrow) of the patient in figure 12 1000 X (Left).

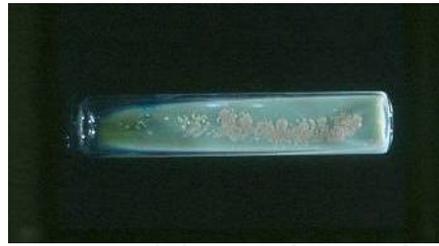


Figure 17. White-yellow colonies of *Mycobacterium chelonae* obtained from the sample of patient in Figure 12.

11. Gram negative bacteria

***Pseudomonas aeruginosa*:** Keratitis caused by *Pseudomonas* that are initiated by the prolonged use of contaminated contact lens, or by traumatic erosion over the corneal epithelium, rapidly progress into cornea stroma and edema in clear cornea zones (Figure 18), these ulcers are painful, and without early antibiotic treatment can progress to endophthalmitis and in some rare cases to severe panophthalmia and vision loss. In the Gram stain of the smear it is observed red small rods (Figure 19), in blood agar white-gray mucous colonies are developed in 18 to 24 hours surrounded by beta hemolytic zone and greenish fluorescein pigment (Figure 20).



Figure 18. *Pseudomonas*, post-trauma keratitis with hypopyon and corneal vessels in unresponsive case to topic antibiotic treatment.

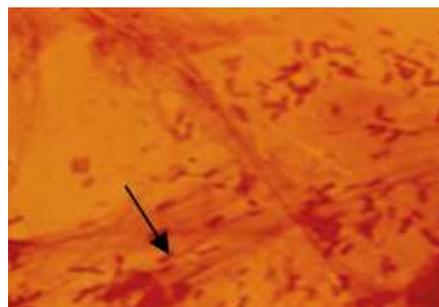


Figure 19. Gram negative rods, (black arrow) in the smear from corneal ulcer from patient in figure 15. 1000 X.



Figure 20. Gray mucous colonies of *Pseudomonas aeruginosa* surrounded by greenish hemolytic zone of fluorescein pigment.

Capnocytophagasputigena. *Capnocytophaga* is a rare keratitis cause, it was described in immuno suppressed patients as risk factor but it was also found in normal young adult patients, (Figure 21). *Capnocytophaga* is normal flora in human and animal (dogs) mouth and the cornea contamination can be by its own patient saliva, in contact lens wearers that use saliva for humectation before putting on his contact lens. In Gram stain smears it appears like negative long rod, (Figure 22) it is a bacterium that grows in 5 to 10% CO₂ environment and move away from the site of seed because it is *flagellae* as is showed in cultures. (Figure 23)



Figure 21. Paracentral, temporal inferior corneal ulceration, caused by *Capnocytophaga sputigena* in a young adult male.

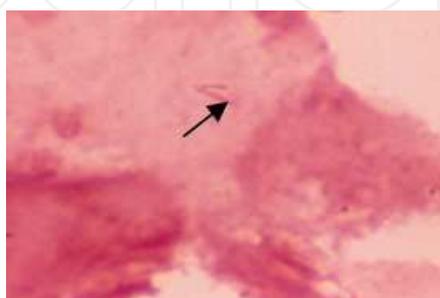


Figure 22. Long and folded Gram negative rod of *Capnocytophaga sputigena* (black arrow) in the secretion keratitis from patient in figure 21



Figure 23. C strikes seeds of *Capnocytophaga sputigena* obtained in the corneal sample culture from patient in figure 21.

Moraxella lacunata. Keratitis related to *Moraxella lacunata* or other *Moraxella* species are observed in patients of any age, in some cases, the authors [19] describe some immunodeficiency in elderly or malnourished people. Keratitis appear like an abscess or like non severe keratitis, with chronic evolution (Figure 24), in the smears Gram and Giemsa stained appear like broad rods (Figure 25) and in culture the colonies are small and translucent (Figure 26), *Moraxella* is a non fermentative bacteria.

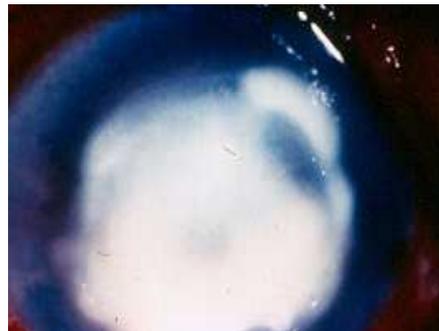


Figure 24. Corneal abscesses in an adult female caused by *Moraxella lacunata*.

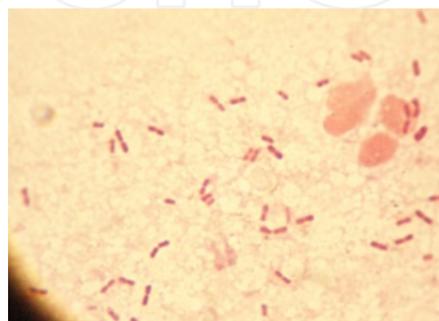


Figure 25. Gram negative broad rods *Moraxella*, from a cornea scraping smear Gram stained. 1000 X.



Figure 26. White-yellow colonies, in the C strikes from cornea samples and conjunctiva samples.

***Neisseria gonorrhoeae*, *N meningitidis*.** Keratitis related to *Neisseria* conjunctivitis or meningitis is always severe and painful, it appears in newborns and in young adults involved in sexual activities. The conjunctiva with unilateral presentation is often observed with a very important edema (chemosis) and for this reason, the patient can no open the eye, after the oral or intravenous administration of adequate antibiotic from betalactamic group, the conjunctiva return to normality and the ophthalmologist can explore searching corneal deep ulcers (Figure 27). An early laboratory diagnostic is mandatory by Gram stain on the abundant conjunctiva secretion, always is observed intracellular Gram negative diplococcic in polymorphonuclear leukocytes (Figure 28), in cultures in agar chocolate and CO₂ ambient colonies are small, gray-translucent, oxidase test positive, and by sugar fermentation, semi automated, automated test or latex coagulation can be specie recognized, and tested for betalactamic antibiotic susceptibility (figure 29).



Figure 27. Deep cornea ulceration near the *limbus*, in a male young adult in a case of *Neisseria gonorrhoeae* kerato conjunctivitis.

12. Fungal keratitis

Mainly ocular trauma, surgical trauma like corneal transplantation (PKP), Laser in situ keratomileusis (LASIK) or Photorefractive keratectomy (PRK), use of contaminated contact lens and dry eye originated from tears alterations are the most common precipitating events for fungal keratitis, some of them are caused by opportunistic white filamentous, melanized, or

yeast like fungus. Early diagnosis and treatment of these chronic and torpid in clinical evolution infections are important, to achieve a better visual acuity.

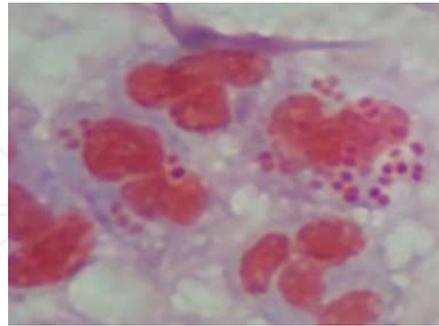


Figure 28. Intracellular in polymorphonuclear leukocyte, Gram negative diplococci observed in conjunctiva secretion in patient from Figure 24 1000X



Figure 29. Antibiogram in Mueller-Hinton Blood agar by diffusion disk method (Kirby and Bauer) of *Neisseria gonorrhoeae*

Some of opportunistic fungus are normal flora in the mouth mucous like *Candida*, others arrive to conjunctiva in the spores forms and do not cause any harmful to conjunctiva or cornea because the normal blink and tear film wash them away [20], the risky factor mentioned above can cause the entrance of the fungi living cells to deep cornea and originate edema and other chronic keratitis with severe clinical manifestations even the ocular loss.

White filamentous Fungi.

Fusarium: *Fusarium solani* are the most frequent cause of keratitis in the series published [21]. In México, 37.2 % fungal keratitis is caused by *F solani*, *F dimerum*, *F oxysporum*, trauma was referred in 35.5 % cases. 75% of cases were observed in males, 38.1% with agricultural activities referred as risk factor, and only 25% in females. In 18 % cases were observed at slit lamp satellite lesions [22], (Figure 30) and 6 % progressed to inflammatory or fungal endophthalmitis, this complication is not frequent in the course of keratitis. [23, 24]

In the laboratory, the diagnosis is made in the scraped sample from the cornea infiltrate as above referred, stained with PAS (Figure 31) or, calcofluor-Evans Blue (Cellfluor) and epifluorescent light microscopy. In the cultures *Fusarium* grows fast, cottony white colonies ap-

pear at 48 or 72 hours in Sabouraud-Emmons at 37° C incubation (Figure 32) [23]. In microcultures for identification, round or piriform microconidia and long-curved macroconidia 3 to 4 cells are characteristic for specie identification.



Figure 30. Satellite lesions, hypopyon in anterior and posterior chamber in a *Fusarium* keratitis in 45 year-old male.

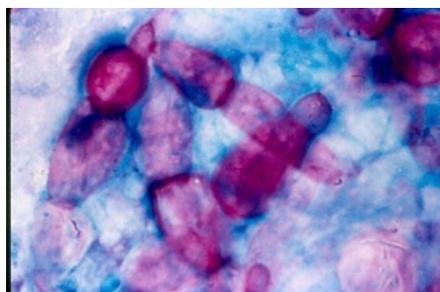


Figure 31. Septate hyphae in corneal scraping smear stained with PAS 1000 X



Figure 32. *Fusarium solani* culture from Figure 30 patient

Aspergillus In keratomycose cases *Aspergillus fumigatus*, *A. nidulans*, *A. flavus*, *A. niger*, and other species are often isolated, corneal infections are severe and with a poor response to antifungal treatment because *Aspergillus* are intrinsically resistant, in Mexico 10.6 % of fungal keratitis was caused by *Aspergillus* in a serial study including 219 cases; 26% patients involved in agricultural activities, 78.6% males and 21.4 females, 26% cases were eviscerated with the ocular loss (Figure 33) [25].

In Sabouraud-Emmons without cicloeximide media growth white greenish-blue or black *Aspergillus niger* colonies in 3 to 4 incubation 27° C days or in blood agar plates (Figure 34), and in microcultures and cotton blue stain shows the characteristic collumela and conidiophores with phialides uniseriate or biseriata with round or oval conidia growing over them (Figure 35).



Figure 33. *Aspergillus flavus* keratitis, three weeks after trauma contaminated with organic soil material.



Figure 34. *Aspergillus nidulans* colony from keratomycose patient in Figure 33



Figure 35. *Aspergillus niger* conidial head, uniseriate phialides and round conidia in lactophenol blue direct microscopic observation from microcultures.

Filamentous Melanized fungus. Many species of opportunistic filamentous melanized (formerly Dematiaceous) fungus related to keratomycoses have been described. *Curvularia*, *Alternaria*, *Phialophora*, *Scyntylydium*, *Cladosporium*, *Scedosporium* in India patients serial studies [26]. In Mexico in a serial patients study of 219 cases 19.1% was caused by melanized fungus [25]. Clinical signs and symptoms seems to keratomycoses caused by white filamentous fungus, (Figure 36) in rare cases the corneal scraping samples shows brown fungal cells (Figure 37), and in microcultures the identification are made by its morphological characteristics.

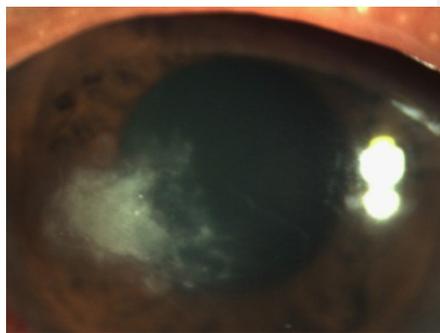


Figure 36. Keratomycose caused by *Curvularia lunata* showing satellite lesions.

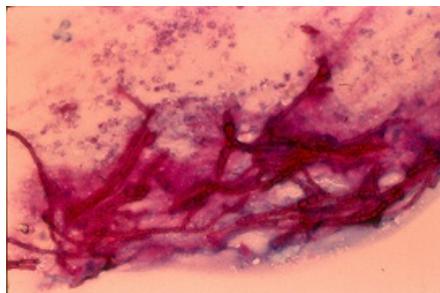


Figure 37. Brown hyphae in cornea smear stained with Schiff periodic acid 400X



Figure 38. *Curvularia lunata* microcultures from the sample of fungal keratitis in the patient of figure 36

Candida. The yeast fungus *Candida albicans*, *C parapsilosis*, *C dubliniensis*, *C tropicalis*, are often isolated from corneal samples in patient with keratitis with post surgical trauma like in cornea transplant, meantime the patient is topically treated with corticosteroids (Figure 39), or in diabetic type 1 or 2 patients. The infiltrate is dense and similar to bacterial keratitis, but without antibiotic treatment response, are indolent and of chronic course, in smears of the corneal secretion can be observed yeast like cells in the PAS stain (Figure 40).

The colonies are obtained in 24 to 48 hours, in blood agar mediums, chocolate agar, Sabouraud-Emmons media, it is suggested to make susceptibility test for Fluconazol and Voriconazole or amphotericin B as recommended by CLSI (Clinical and Laboratory Standard Institute)

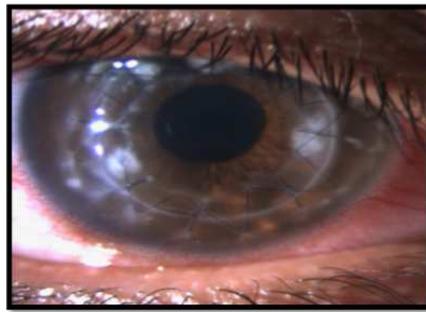


Figure 39. Candida keratomycose in a young male after penetrating keratoplasty for keratoconus

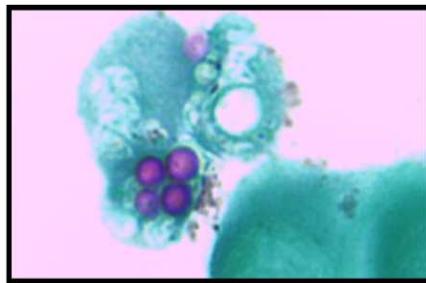


Figure 40. Buddy yeast like cells stained with PAS from corneal smear in patient in Figure 39 1000 X.



Figure 41. Creamy-white colonies of *Candida tropicalis* in Sabouraud media.

13. Conclusion

Bacterial or fungal corneal inflammation or ulcerations are threatening condition for visual function; the early and accurate diagnosis and specific medical treatment are the gold standard to achieve the best prognosis.

Keratitis caused by Gram negative bacteria and a fungal keratitis clinically seems very similar by clinical signs, the support that the laboratory of microbiology can give for the differentiation between two entities are very important because the medical treatment are made with different drugs, in the other hand keratitis caused by Gram positive bacteria and yeast like fungus, are very similar in inflammatory signs, and in this cases one smear can make the differentiation.

In *Neisseria gonorrhoeae* keratitis, one delayed or not laboratory confirmation diagnosis is of high risk because the corneal tissue loss is always important and there is risk to lose the whole cornea.

Other options for detection of yeast or filamentous fungi in corneal samples are PCR techniques as suggest Baine using 18S rRNA: 28S rRNA or ITS PCR [27] when the patient has been previously antifungal treated and the corneal smears and cultures are negative for fungal cells search, he suggest PCR as complementary test for traditional cultures as mentioned above. Goldschmidt et al. using High-resolution melting technique [28], detected and differentiated yeast like and filamentous fungi in keratomycose samples in 46 patient in a more simple, specific and cost-efficient test. In 10 negative culture samples they detected 7 cases positive for fungal infections. We have no experience in mycotic keratitis detected by PCR or PCR derived techniques, never the less in one culture proved *Histoplasma capsulatum* scleritis [29].

It is very important for the best prognosis in keratitis cases, to confirm the clinical diagnosis by the laboratory work since the first consultation, for to start immediately the specific medical topical treatment.

For all those reasons the laboratory support in the clinical diagnosis of keratitis is very important in order to achieve a shorter evolution time and to achieve a small scar for the better visual acuity in a patient suffering for a corneal infection.

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Author details

Virginia Vanzzini Zago* and Ana Lilia Perez-Balbuena
*Address all correspondence to: vivanzzini@yahoo.com

Laboratory of Microbiology, Hospital Asociación Para Evitar la Ceguera en México "Dr Luis Sánchez Bulnes", México City, Mexico

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