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Bacterial Keratitis Infection: A Battle Between Virulence Factors and the Immune Response

Atzin Robles-Contreras, Hector Javier Perez-Cano,
Alejandro Babayan-Sosa and Oscar Baca-Lozada

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1. Introduction

1.1. Epidemiology

Microbial keratitis is a potentially serious corneal infection and a major cause of visual impairment worldwide. A conservative estimate of the number of corneal ulcers occurring annually in the developing world alone is 1.5–2 million [1]. The incidence of this condition varies from 11.0 per 100 000 person years in the United States to 799 per 100 000 person years the developing nation of Nepal [2, 3]. Microbial keratitis is thus a significant public health problem, and numerous studies have been performed describing the microbiology of corneal infection. Wide geographical variation exists in the epidemiology of microbial keratitis based on economic and climate factors. To some degree, this variation is explained by economic factors as well as contact-lens wear. A high proportion of bacterial ulcers were reported from centres in developed countries (North America, Australia and Western Europe). In these countries, patients are far less likely to be agricultural workers, and so have a reduced risk of trauma from organic matter, which is known to be a risk factor for fungal infection.

Almost any microorganism can invade the corneal stroma if the normal corneal defense mechanisms are compromised. A wide spectrum of microbial organisms can produce corneal infections and, consequently, the therapeutic strategies adopted for its treatment may be varied. As there is no definite pathognomonic clinical feature, it is difficult to establish the aetiology of corneal ulcer merely on the basis of clinical features. Hence, microbiological evaluation is a must in order to attain a definitive diagnosis and to ensure specific therapy for keratitis.

Regarding bacterial keratitis there are several potential risk factors such as contact lenses, trauma, aqueous tear deficiencies, neurotrophic keratopathy, eyelid alterations or malposition, decreased immunologic defenses, use of topical corticoid medications and surgery [4]. Trauma is a major risk factor for corneal infection in developing countries. In Paraguay, the percentage of cases with preceding trauma was 48%, in Madurai, South India, 65% and 83% in Eastern India [5, 6, 7]. By far the most common cause of trauma to the corneal epithelium and the main risk factor for bacterial keratitis in developed countries is the use of contact lenses, particularly extended-wear contact lenses. Patients with bacterial keratitis, 19-42% are contact lens wearers; incidence of bacterial keratitis secondary to use of extended-wear contact lenses is about 8,000 cases per year. The annual incidence of bacterial keratitis with daily-wear lenses is 3 cases per 10,000 [8].

Traditionally the more common groups responsible for bacterial keratitis are: *Streptococcus* sp., *Pseudomonas* sp., *Enterobacteriaceae* (including *Klebsiella*, *Enterobacter*, *Serratia*, and *Proteus*), and *Staphylococcus* sp. Although there is also a wide variation depending on the setting of the series reported. A high percentage of *Staphylococcus* sp. (79%) was recorded in a study from Paraguay, although the reason for this is not clear. Another study found the highest proportion of *Streptococcus* sp. (46.8%), the authors noted that this figure was only 18.5% in 1986 and suggest that the trend might represent a genuine change in the bacterial flora owing to changes in the climate and environment [9]. A study from Bangkok [10] had the highest proportion of *Pseudomonas* infections (55%). Interestingly, this study did not have the highest proportion of contact-lens wearers. Other studies reported far higher proportions of contact-lens wearers—for example, 44% in a study from Taiwan [11] and 50% in a study from Paris [12]. When compared the percentage of contact-lens wearers with the percentage of pseudomonal infections, the Spearman correlation coefficient was not statistically significant. Cohen et al. at Wills Eye Hospital reported a decline in contact lens-related ulcers: during 1991 to 1998, contact-lens wear accounted for 44% of all ulcers, but during 1992 to 1995, it accounted for only 30%. Liesegang reports the following risk factors for development of bacterial keratitis among contact lenses wearers: overnight wear, smoking, male sex, and socioeconomic status. The risk with therapeutic contact lenses is much higher: approximately 52/10,000 per year [13].

Jeng [14] commented on the emerging resistance of bacterial infections to fluoroquinolones. In addition to changes in resistance patterns, studies have also demonstrated changing patterns of causative organisms over time in a given geographical location. Varaprasathan et al [15] reported that the proportion of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* ulcers in Northern California had decreased over a 50-year period, while that of *Serratia marcescens* had increased over the same period. Sun et al [16] reported a rise in the percentage of Gram positive (+) cocci in North China from 25% in 1991 to 70.8% in 1997, as well as a decrease in Gram negative (-) bacilli from 69% to 23.4% over a similar period. Hsiao et al [17] reported on a 10 year follow up that there was a significant decrease in the percentage of Gram(+) microorganisms over time. The sensitivity of Gram(-) isolates to tested antimicrobials was >97% response for all the reported antibiotics; this was not the case for Gram(+) isolates, in which resistance to the antibiotics was more common, methicillin-resistant organisms accounted for 29.1% of all Gram(+) cultures.

The overview of bacterial keratitis is quite extensive, from the epidemiological point of view is important to consider the wide variety of presentations even within the same regions of a country. Microbiological studies are essential to determine the casuistry of each center in a given time. A common problem throughout the world is the ever increasing resistance to antibiotics including the new fluoroquinolones.

2. Immune response

We have a lot of mechanisms to evade a bacterial infection: physical, chemical, microbiological, and immunological mechanisms. But not all of the mechanisms are described in a bacterial keratitis infection.

2.1. Exterior defense

The eye has several mechanisms to prevent colonization by bacteria, among which are three main types: the mechanics, such as blinking, or that the Tight Junctions present in the corneal epithelial cells, preventing the entry of bacteria into the corneal stroma or other intraocular structures, it is important to mention that certain bacteria are able to penetrate the intact corneal epithelium such as *Corynebacterium diphtheriae*, *Haemophilus aegyptus*, *Neisseria sp.*, *Listeria sp.* and *Shigella sp.* [18, 19]; Chemicals, which are the presence of soluble molecules involved in controlling the growth of bacteria, such mechanisms are presence of lactoferrin, lysozyme, antimicrobial peptides, antibodies, etc. [20-21] and finally microbiologic mechanisms, these mechanisms refer the normal microbiota of the ocular surface (*S. epidermidis*, *S. aureus* and *Propionibacterium sp.*) [22-26], the microbiota generates substances called bacteriocins, which will be mentioned later in item 3 of this chapter.

2.2. Complement

It was reported in murine models, that anaphylatoxins (C3a, C4a, and C5a) could be generated when the cornea was injured with lipopolysaccharides (LPS), immune complexes, acid, or alkali. Interestingly membrane attack complex (MAC) could only be generated when the cornea was exposed to LPS or immune complexes. Cornea failed to generate MAC when confronted with acid or alkali. The immune response mounted to LPS or immune complex is similar to that generated against infectious agents like Gram(-) bacteria. Indeed the complement system has been shown to play a critical role in protection against *Pseudomonas aeruginosa* infection that causes keratitis [27,28]. Additionally, complement activation is believed to play an important role in ulceration of human cornea induced by Gram(-) bacteria [29].

2.3. Receptors

There are different receptors that recognizes bacteria molecules, these receptors in general are called pattern recognition receptors and exists several types of receptors (TLR, CLR, NLR and RLR). In bacterial keratitis infections are studied in murine and *in vitro* models, the

presence, activation and function of TLR. The functions described in TLR activation are cytokine secretion, chemokines secretion, and antimicrobial peptides secretion, recruitment of cells to inflammation site. For example corneal TLR4 expression is increased in *P. aeruginosa* infection and deficiency of this receptor in BALB/c mice resulted in a susceptible rather than resistant phenotype [30], these observations suggest that TLR4 is critical for resistance to *P. aeruginosa* keratitis.

2.4. Effects of receptors activation

2.4.1. Chemokines

In other studies, UV killed *S. aureus* and Pam3Cys (TLR2 synthetic ligand) stimulated the phosphorylation of MAP kinases, JNK, p38 MAPK and ERK, and the blockade of JNK, but not that of p38 or ERK phosphorylation, had an inhibitory effect on I κ B α degradation and CXC chemokine production [31]. Furthermore they also found that corneal inflammation was significantly impaired in mice deficient in JNK1 mice compared with control mice, suggesting that JNK has an essential role in TLR2-induced corneal inflammation.

2.4.2. Antimicrobial peptides

Activation with pathogens and TLR agonists of ocular surface epithelial cells by also leads to the production of antimicrobial peptides such as hBD-2 and the cathelicidin LL-37 [32-34]. In an interesting *in vitro* study [35], Maltseva et al., reported that a MyD88 dependent increase in corneal epithelial hBD-2 expression caused by exposure to *P. aeruginosa* supernatant was abrogated by the presence of a contact lens, thus giving new insight into the mechanism by which contact lens wear predisposes to *P. aeruginosa* keratitis.

Additional *in vivo* studies have shown that defensins and LL-37 play an important role in protecting the ocular surface from *P. aeruginosa* infections. In particular, mice deficient in cathelicidin-related antimicrobial peptide (CRAMP), the murine homologue of LL-37, are more susceptible to *P. aeruginosa* keratitis, had significantly delayed bacterial clearance and an increased number of infiltrating neutrophils in the cornea [36]. A similar finding was reported in BALB/c mice following knock down of mBD-2 or mBD-3, but not of mBD-1 or mBD-4, by siRNA [37-38]. Furthermore Wu et al. also found that silencing mBD2, mBD3 or both defensins resulted in a significant upregulation of TLR2, TLR4 and MyD88 but not TLR5 or TLR9 [39].

Kumar et al. [40] observed that pre-treatment with the TLR5 agonist flagellin markedly reduced the severity of subsequent *P. aeruginosa* infection in C57BL/6 mice. This was in part due to induction of corneal expression of the antimicrobial molecules, nitric oxide and CRAMP. They also observed similar results *in vitro*, as flagellin pre-treatment enhanced *P. aeruginosa* induced expression of hBD-2 and LL-37 in human corneal epithelial cells [39]. These observations raise the possibility of utilizing TLR activation as a prophylactic means of preventing an overwhelming inflammatory response and corneal destruction in *P. aeruginosa* keratitis.

2.5. Recruited cells

2.5.1. Polymorphonuclear cells

In animal models as characterized by bacterial invasion of the underlying stroma and intense neutrophil infiltration which results in corneal opacification and potentially loss of vision [41-45]. In an murine model of *S. aureus* keratitis, exposure of corneal epithelium to *S. aureus* increased neutrophil recruitment to the corneal stroma, corneal thickness and corneal haze in normal C57Bl/6 mice, mice deficient TLR4 or TLR9, but not in mice deficient in TLR2 or MyD88, suggesting that *S. aureus*-induced corneal inflammation is mediated by TLR2 and MyD88 [46].

In 2005 Huang et al., reported that silencing TLR9 by siRNA in C57BL/6 mice resulted in less severe inflammation, reduced polymorphonuclear infiltration but consequently increased bacterial load [47]. These data suggested that TLR9 activation is required to adequately eliminate bacteria but that it also contributes to corneal destruction.

2.5.2. T cell populations

Extensive study of the underlying mechanism of the pathogenesis of *P. aeruginosa* keratitis in experimental models has revealed that mice can be divided in two groups based upon their immune response to the pathogen [48]. BALB/c mice are resistant to *P. aeruginosa* infection as they mount a Th2 based response that facilitates recovery and corneal healing. While C57BL/6 mice are susceptible to *P. aeruginosa* infection as they mount a Th1 based immune response leading to corneal perforation. Comparison among these mouse strains provides a unique opportunity to understand the immune response to *P. aeruginosa*.

Exists other type of effectors in the immune response not characterized yet, like the presence of other receptors like NLR or CLR. It is important to mention that the immune response previously described are in animal models or *in vitro* models; a few studies are in patients and we need to study in the future to explain the immunopathogenesis and found new treatments for patients.

3. Virulence factors and mechanisms of bacterial resistance

To understand why bacterial keratitis is often of difficult treatment is necessary to first review the virulence factors and mechanisms of bacterial resistance, this will help us to make decisions about treatment, patient management and contribute to prevent the emergence and development resistant strains.

The treatment for bacterial keratitis consist mainly in antibiotics, so it is necessary to know: bacterial structure, biochemical action, identified important immunogens, and virulence factors. Molecular biology also has had a great participation and that made possible the development of molecular techniques with applications to research to learn more about the

bacterial virulence factors and in the diagnosis of pathogens to give a prompt and timely treatment [49].

3.1. Virulence factors

Virulence is a term that comes from the Latin virulent (virus = poison and virulent = poisonous) and that is a property that allows pathogenic bacteria to colonize the host and thus obtain their nutritional requirements, for this it is necessary to evade the defense mechanisms, multiply, establish and cause harm. All this is achieved through the expression of bacterial virulence factors (bacteriocins) that allow microbial adherence, invasion, or both, the harmfulness and pathogenic microorganism determines its virulence or their ability to do harm. Within the virulence factors we can mention the following:

- **Adhesins.** These substances are membrane receptors involved not only in the cell-cell interactions but also cell-extracellular matrix and cell-trafficking cell. Among the adhesins find bacterial pili, fimbrial proteins, lipoteichoic acids and glycocalyx.
- **Invasins.** Surface proteins which are responsible for reorganization of actin filaments near the cytoskeleton, thus, when a bacterium comes into contact with the host cell occurs a change in its structure similar to a drop of liquid on a solid surface falls due the reorganization of the cytoskeleton so that it can be incorporated into the cell, once inside the bacteria uses actin to move from one cell to another.
- **Impedins.** Molecules that help the bacteria evade the host immune response to perpetuate and maintain their infectivity, as examples we can mention the mucinases that using mechanical effects generated by the movement of flagella prevent skidding and disposal, also we can mention proteases that are found mainly in the mucous membranes and destroy the IgA antibodies. In addition exist molecules that help evasion of phagocytosis as coagulase, DNase, phosphatases, LPS that interfere with complement and finally the production of toxic metabolites to overcome the normal flora.
- **Aggressins.** Hypothetical substance held to contribute to the virulence of pathogenic bacteria by paralyzing the host defensive mechanisms which, by their chemical nature, can lead to tissue damage, inflammation and shock. Some examples we can cite alpha and beta toxins, lytic enzymes, DNases, lipases, hyaluronidases, kinases, teichoic acid.
- **Modulins** are bacterial components that promote the production of cytokines among which we can find the lipopolysaccharide of Gram(-), superantigens and murein fragments.

3.2. Bacterial resistance

The principal objective about the study of bacterial virulence factors is the quest for new preventive and therapeutic tools against many infectious diseases. However, there is another condition called bacterial resistance[50].

Antibiotic resistance in bacteria has become a health problem worldwide. The developments of new antibacterial drugs, the indiscriminate and irrational use, besides the evolutionary

pressure exerted by therapeutic use have gone masking the increase of the resistance. It appears that the design or discovery of new antibiotics solve the problem, however, also new mechanisms of resistance are difficult to control

Infections caused by multiresistant bacteria, causing extensive morbidity and mortality and the cost per hospitalization and complications is high. The selective pressure plays an important role in the occurrence of resistant strains and is favored by free prescription and formal therapeutic use, the widespread use of antimicrobials in immunocompromised patients, in the intensive care unit, the use of inadequate dose or insufficient duration of antimicrobial therapy and indiscriminate use without establishing a profile sensitivity of isolates. The selective pressure is a process of adaptation and this is not an attribute of individual organisms or nature or life, but it is attributes of a species. In Darwinian terms, the response to the selective pressure is not the individual, not life or nature as a whole but the population itself [51], this means that when a treatment is handled improperly, only susceptible organisms will be destroyed and reduce the bacterial load and hence the infection symptoms, however resistant microorganisms remain in small amounts and gives rise to a new generation of resistant strains (figure 1).

Figure 1. Selective pressure after a treatment with dosage, time or inadequate concentration of the antibiotic. (A) mixture of sensitive and resistant bacteria to an antibiotic (B) Resistant bacteria (C) Proliferation of bacteria resistant proliferation.

The phenotypic expression of bacterial resistance has intrinsic or acquired genetic basis and is mainly expressed by biochemical mechanisms [52]. Briefly describe the two mechanisms of bacterial resistance, the naturally occurring and acquired by the same bacteria.

3.2.1. Natural resistance

Natural resistance is a constant feature of strains of the same bacterial species and is a permanent mechanism determined genetically and furthermore correlated with dose of antibiotic. Some examples of this, we can mention the resistance presented by *Proteus mirabilis* to the tetracyclines and colistin, *P. aeruginosa* to the Benzylpenicillins and trimethoprim-sulfamethoxazole, aerobic Gram(-) bacilli to the clindamycin, *Klebsiella pneumoniae* to the penicillins (ampicillin and amoxicillin), [53].

3.2.2. *Acquired resistance*

Bacterial species, which by nature is sensitive to an antibiotic, can be genetically modified either by mutation or by acquisition of resistance genes (plasmids, transposons and integrons), these are evolutionary and their frequency depends on the use of antibiotics. An example of mutation of a gene involved in the mechanism of action of an antibiotic is the DNA gyrase involved in DNA replication process of enterobacterias and that a mutation in these genes can confer resistance to quinolones; can also be mutations generated in genes encoding the porins which results in blocking the entrance of the antibiotic into the microorganism. The acquisition of resistance genes can be obtained by transfer from a strain of a species identical or different, mechanisms responsible for these are the plasmids, transposons and integrons [53-54].

The plasmids and transposons are mobile genetic elements which carry resistance genes. The plasmids are fragments of bacterial DNA with variable length; some have the ability to replicate independently of the genetic machinery available to the cell. Other hand transposons are sequences of DNA (double stranded) which can be translocated from chromosome to chromosome or a plasmid to plasmids, thanks to a proper recombination system, this adds to the ability of plasmids to move from one cell to another during conjugation, this allows the acquisition of resistance genes from bacteria of the same species or different species which facilitates the expansion of the resistance strains. Some plasmids and transposons have elements called integrons gene that allows them to capture more exogenous genes determining the development of resistance to several antibiotics (multiple resistance). Antibiotics particularly affected by this mechanism are the beta-lactams, aminoglycosides, tetracyclines, chloramphenicol, and sulfonamide, an example is the resistance presented by *Escherichia coli* and *P. mirabilis* to ampicillin [55].

3.3. Resistance mechanisms

Bacterial resistance both acquired and natural can be approached from the standpoint molecular and biochemical and can be classified into three basic mechanisms of resistance expressed according to the mechanism expressed and the antibiotics mechanism action and may occur simultaneously [55]. The figure 2 shows a schematic representation of the mechanisms of resistance.

- Inactivation of antibiotic by destruction or modification of chemical structure. Is a molecular process characterized by the production of enzymes that carry out this function. For example, enzymes that destroy the chemical structure of an antibiotic against beta-lactamases are characterized by hydrolyzing the beta-lactam nucleus through amide bond cleavage and erythromycin esterase which catalyses the hydrolysis of the lactone ring of the antibiotic, while the enzymes responsible to the modification of the structure we can mention the chloramphenicol acetyl transferase, enzymes that modify aminoglycosides, lincosamides and streptogramins, other enzymes belonging to this group are acetylases, adenilases and phosphatases [56, 57].

- Altered target site of the antibiotic. Is the modification of bacterial specific sites such as the cell wall, cell membrane, or both 30S and 50S ribosomal subunit. The modification by mutation of GyrA and GyrB genes that coding for topoisomerase II and IV offer bacterial resistance to *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli* to quinolones [57]. Among the ribosomal level changes can include changes in the 30S and 50S subunits which are sites of action of aminoglycosides, macrolides, tetracyclines and lincosamides. Methylation of ribosomal RNA from the 50S subunit confers resistance to *S. aureus* and *S. epidermidis* against tetracycline, chloramphenicol and macrolides. Mutation of the 30S subunit confers resistance to gentamicin, tobramycin and amikacin [55,58].
- Altered permeability barriers. Is due to specific changes in structure of antimicrobial receptors or alterations in the components of the wall or cell membrane and occur changes in the permeability, as well as the loss of the ability of active transport across the cell membrane or the expression of efflux pumps which are activated at the time that the antibiotic is introduced into the bacterial cell [53]. The internalization of hydrophilic compounds is carried out by channels called porins which are filled with water, penetration of the antibacterial in this case depend on the size of the molecule, hydrophobicity and electric charge [55].
- Efflux pumps: On the cell membrane are efflux pumps that carry out the internalization and removal of antimicrobials, a wide variety of these provide antimicrobial resistance both Gram(+) and Gram(-). Active efflux of antibiotics is mediated by transmembrane proteins and the Gram(-) bacteria, involves the membrane components and cytoplasm. These proteins are exported active channels to an antimicrobial agent outside the cell as fast as it comes. These mechanisms confer resistance to tetracycline, quinolones, chloramphenicol, beta lactam antibiotics, antiseptics and disinfectants quaternary ammonium type used for cleaning surfaces [53, 55, 57, 58].

3.4. Biofilm production

In nature, bacteria can grow like planktonic or free-floating, but can also grow colonies embedded in a matrix known as biofilm. Deserves special mention the formation of biofilms, since being a microbial ecosystem composed of one or more microorganisms associated with living or inert surface with functional features and complex structures can be considered a virulence factor and the same time a resistance mechanism. Biofilm formation enables the adhesion to the surface where the bacteria is present and can be one of many causes of chronic infections, for example the chronic infectious keratitis. The structural organization of the bacterial biofilm is composed of polysaccharides, nucleic acids and proteins and all this set is known as extracellular polymeric substances (EPS) and its production is affected by the nutritional quality of the environment in which bacteria develop when the environment is suitable to form biofilms with multiple microcolonies, so the structure that forms is so great that prevents phagocytosis and effects of the immune system against them, for this reason is considered a virulence factor. A very important advantage from the clinical point of view is that biofilms confer resistance to antibiotics such that the dose can be increased thousands of times without causing damage [59]. Two hypotheses to explain the resistance

generated by the production of biofilms, the first indicating that occurs a limited penetration of the drug and the bulk is left on the surface such that the antibiotic never reaches its target. The second refers to the physiological limitation and proposes that some microorganisms within the biofilm can exist in a more recalcitrant phenotypic state. Anderl JN et al [60] in a study of *K. pneumoniae* found that the planktonic form was sensitive to ampicillin and reported minimum inhibitory concentration (MIC) of 22µg/mL while the same strain that grew as a biofilm presented a survival of 66% increasing the concentration of ampicillin to 5000µg/mL which corresponds to 2500 times the MIC.

Figure 2. Resistant mechanism (1) Altered target site of the antibiotic and altered permeability barriers (2) Inactivation of antibiotic by destruction or modification of chemical structure (3) Efflux pumps (4) acquisition of resistance genes by fagos (5) Plasmids (6) Transposons and Integrons (7) modification by mutation of topoisomerase.

Having recognized the role of biofilm as responsible of infectious diseases, it is necessary the search for new approaches in both the treatment and prevention. A proposal to counteract this resistance factor is the alteration of the surface to inhibit adhesion. In the area of oph-

thalmology, for example, chelating agents could be used in contact lens solutions, mainly iron-trapping agent which is necessary for adhesion of the pili of *Pseudomonas sp.* [59, 60].

Figure 3. Biofilm production (1) Planktonic bacteria encounter a submerged surface. They begin to produce slimy extracellular polymeric substances (EPS) and to colonize the surface. (2) EPS production allows the emerging biofilm community to develop a complex three-dimensional structure (3) Biofilms can propagate through detachment of small or large clumps of cells that releases individual cells.

4. Clinical characteristics

4.1. Common characteristics

When developing a bacterial corneal ulcer usually appears chemosis and conjunctival injection, eyelid edema, decreased vision, pain, tearing, photophobia, and purulent discharge. Conjunctival reaction is nonspecific, with a predominantly papillary response, is primarily limbal injection. The corneal epithelium and stroma ulcer shows a gray-white infiltrate, may appear necrotic. Infiltration and edema of the cornea can be observed even in areas remote from the ulcer. Appears frequently, an anterior chamber reaction, and in severe cases can be observed fibrin plates on the endothelium and may be a fibrinoid aqueous or hypopyon [61-64].

The hypopyon is produced by the toxic effects of infection on vessels iris and ciliary body, with consequent pouring of fibrin and polymorphonuclear leukocytes. Usually, the hypopyon is sterile as Descemet's membrane is intact. Hypopyon can be seen with any bacterial infection, most frequently in ulcers caused by *S. pneumoniae* and *Pseudomonas sp.*; not forgetting also can occur in viral and fungal ulcers [64-66].

Signs and symptoms of bacterial corneal ulcers vary depending on the virulence of the organism, the previous state of the cornea, the duration of infection, host immune status and prior use of antibiotics and steroids [67-70]. The use of hydrophilic contact lenses can alter the presentation of bacterial ulcers. Infections associated with contact lenses are often multifocal and epithelial and stromal infiltrate is more diffuse. The contact lens wearers presenting with corneal abrasions may have bacterial infections early[71, 72]. Figure 4 are clinical pictures representative in bacterial keratitis.

The aspect sometimes ulcer suggesting the presence of a specific bacterial agent or a group of them. Are indicated below the characteristic signs of infection caused by some agents. However, one must take into account the clinical aspect is never diagnosis, isolation and identification of causative agents is always essential.

4.2. *Staphylococcus sp.*

S. aureus produces coagulase and mannitol fermentation being more aggressive, and *S. epidermidis* does not produce coagulase or ferment mannitol. The latter two are usually opportunistic pathogens that cause infections in compromised corneas, for example, persistent epithelial defects, bullous keratopathy, herpetic epitheliopathy, diabetic epitheliopathy, etcetera. The corneal appearance in *S. aureus*, has a round or oval ulcer, localized, with distinct edges and tends to be deeper, usually accompanied by a creamy white stromal infiltrate and well-defined gray with overlying epithelial defect and can be multifocal. In severe cases, you can get to see hypopyon and endothelial plaque, staphylococcal blepharitis is common [73-75].

Figure 4. Representative Clinical pictures of patients with Bacterial Keratitis infection..

4.3. *Pseudomonas sp.*

Pseudomonas sp. is a Gram(-) often associated with contact lens use, which adheres to the damaged epithelium and stromal cause rapid invasion, the ulcer has a deep peripheral extension in hours (can reach twice its size in 24 hrs.) peripheral infiltration with diffuse gray, yellow-green discharge, severe reaction and hypopyon in the anterior chamber, which may extend to sclera and cause necrotizing scleritis and/or perforation in 2-5 days. You can also get to see a multifocal pattern that is more associated with use of soft contact lens [67, 76].

4.4. *Streptococcus sp.*

S. pneumoniae isolates in the upper respiratory tract half of the population, their proximity to the eye may explain the frequency of problems associated with it. *Streptococcus sp.* generates hemolysis of erythrocytes, being in full by the *S. pyogenes*, and partially by the alpha hemolytic as *S. viridans* and *S. pneumoniae*. The infection usually arises after corneal trauma and is often associated with chronic dacryocystitis. We present a deep stromal abscess with fibrin deposition, plaque formation, severe anterior chamber reaction, hypopyon, synechiae iridiana, if left untreated can lead to perforation. *S. viridans* has a less aggressive course and is responsible for cristalinean keratopathy, also related to the indiscriminate use of topical anesthetic, use of contact lenses and chemical burns [77-79].

4.5. *Bacillus sp.*

Bacillus cereus are bacilli anaerobic Gram + in soil, water and vegetation. The infection usually occurs within 24 hours after penetrating trauma in the presence of chemosis, severe eyelid edema, proptosis, edema peripheral microcystic with a ring followed by a circumferential corneal abscess may lead to drilling in hours [80-82].

4.6. Less common infectious agents

4.6.1. *Corynebacterium diphtheriae*

Corynebacterium diphtheriae are bacilli Gram + that rarely causes corneal disease but does remark commonly as a cause of pseudomembranous conjunctivitis with preauricular lymphadenopathy resulting in corneal epithelial opacity diffuse stromal necrosis and thinning [83-87].

4.6.2. *Listeria monocytogenes*

Listeria monocytogenes is a facultative anaerobe that causes infection in people who are dedicated to animal care. It is colonizing persistent epithelial defects and keratitis developed a type of necrotizing ulcer-shaped ring with large anterior chamber reaction, fibrinoid exudation and hypopyon [87-89].

4.6.3. *Propionibacterium acnes*

Propionibacterium acnes are bacilli anaerobic Gram + rod that is part of normal flora, so the infection occurs before a surgical trauma, contact lens use, chronic use of steroids or other associated corneal disease. It takes the form of corneal stromal abscess with intact epithelium [90-93].

5. Conclusions

In order to minimize the effect of bacterial resistance have begun to develop programs among which we mention the use of antibiotics, increased medical education plans in the study of infectious diseases, the use of antimicrobial agents and their prescription based on the evidence, the establishment of surveillance programs to detect the emergence of resistant strains, and improving the quality of antimicrobial susceptibility methods.

In the future it will continue to develop new antibiotic molecules looking to have a better effect. However, we must control a number of factors that facilitate the increase and acceleration of development of resistance, it is necessary to continuously monitor the levels of resistance of each bacterial species and thus able to make a rational antibiotic selection for the benefit of patients and reduce the risk of developing resistance. Simple measures and common sense will remain the main limiting resource for development of bacterial resistance.

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Author details

Atzin Robles-Contreras¹, Hector Javier Perez-Cano¹, Alejandro Babayan-Sosa² and Oscar Baca-Lozada²

1 Biomedical Research Center, "Nuestra Señora de la Luz" Hospital Foundation, Mexico

2 Cornea Department, "Nuestra Señora de la Luz" Hospital Foundation, Mexico

References

- [1] Whitcher JP, Srinivasan M & Upadhyay MP. Corneal blindness: a global perspective. *Bull World Health Organ* 2001; 79:214-21.
- [2] Erie JC, Nevitt MP, Hodge DO & Ballard DJ et al. Incidence of ulcerative keratitis in a defined population from 1950 through 1988. *Arch Ophthalmol* 1993; 111:1665-71.
- [3] Upadhyay MP, Karmacharya PC, Koirala S, Shah DN, Shakya S, et al. The Bhaktapur eye study: ocular trauma and antibiotic prophylaxis for the prevention of corneal ulceration in Nepal. *BrJ Ophthalmol* 2001;85:388-92.1997; 104:1902-9.
- [4] Green M, Apel A & Stapleton F. Risk factors and causative organisms in microbial keratitis. *Cornea* 2008 Jan; 27:22-7.
- [5] Laspina F, Samudio M, Cibils D, Ta CN, Fariña N, et al. Epidemiological characteristics of microbiological results on patients with infectious corneal ulcers: a 13-year survey in Paraguay. *Graefes Arch Clin Exp Ophthalmol* 2004; 242:204-9.
- [6] Srinivasan M, Gonzales CA, George C, et al.. Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, south India. *Br J Ophthalmol* 1997; 81:965-71.
- [7] Basak SK, Basak S, Mohanta A, et al. Epidemiological and microbiological diagnosis of suppurative keratitis in Gangetic West Bengal, eastern India. *Indian J Ophthalmol* 2005; 53:17-22.
- [8] Cohen EJ, Fulton JC, Hoffman CJ, et al. Trends in contact lens-associated corneal ulcers. *Cornea* 1996; 15:566-70.
- [9] Shah A, Sachdev A, Coggon D, et al. Geographic variations in microbial keratitis:an analysis of the peer-reviewed literature. *Br J Ophthalmol*. 2011; 95:762-7.
- [10] Sirikul T, Prabripataloong T, Smathivat A, et al.. Predisposing factors and aetiologic diagnosis of ulcerative keratitis. *Cornea* 2008; 27:283-7.
- [11] Fong CF, Tseng CH, Hu FR, et al.. Clinical characteristics of microbial keratitis in a university hospital in Taiwan. *Am J Ophthalmol* 2004;137:329-36
- [12] Bourcier T, Thomas F, Borderie V, et al. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. *Br J Ophthalmol* 2003;87:834.
- [13] Liesegang TJ. Contact lens-related microbial keratitis: Part I: Epidemiology. *Cornea* 1997;16:125-31.
- [14] Jeng BH & McLeod SD. Microbial keratitis. *Br J Ophthalmol* 2003;87:805-6.
- [15] Varaprasathan G, Miller K, Lietman T, et al.. Trends in the aetiology of infectious corneal ulcers at the F.I. Proctor Foundation. *Cornea* 2004;23:360-4
- [16] Sun X, Deng S, Li R, et al. Distribution and shifting trends of bacterial keratitis in north China . *Br J Ophthalmol* 2004;88:165.

- [17] Hsiao CH, Chuang CC, Tan HY, et al. Methicillin-resistant *Staphylococcus aureus* ocular infection: a 10-year hospital-based study. *Ophthalmology*. 2012 Mar; 119(3): 522-7.
- [18] Yang KS, Lin HC, Ma DH, et al. Ulcerative keratitis caused by *Haemophilus influenzae*. *Cornea*. 2006 Jul;25(6):701-4.
- [19] Tjia KF, van Putten JP, Pels E, et al. The interaction between *Neisseria gonorrhoeae* and the human cornea in organ culture. An electron microscopic study. *Graefes Arch Clin Exp Ophthalmol*. 1988;226(4):341-5.
- [20] Zhou L, Zhao SZ, Koh SK, et al. In-depth analysis of the human tear proteome. *J Proteomics*. 2012; 75(13):3877-85. Epub 2012 May 23.
- [21] Zhou L & Beuerman RW. Tear analysis in ocular surface diseases. *Prog Retin Eye Res*. 2012 Jun 23. [Epub ahead of print].
- [22] Keay L, Willcox MD, Sweeney DF, et al. Bacterial populations on 30-night extended wear silicone hydrogel lenses. *CLAO J*. 2001; 27(1):30-4.
- [23] Leitch EC, Harmis NY, Corrigan KM, Willcox MD. Identification and enumeration of staphylococci from the eye during soft contact lens wear. *Optom Vis Sci*. 1998; 75(4): 258-65.
- [24] Leitch EC, Willcox MD. Interactions between the constitutive host defences of tears and *Staphylococcus epidermidis*. *Aust N Z J Ophthalmol*. 1997 ;25 Suppl 1:S20-2.
- [25] Hart DE, Hosmer M, Georgescu M, et al. Bacterial assay of contact lens wearers. *Optom Vis Sci*. 1996; 73(3):204-7.
- [26] Ramachandran L, Sharma S, Sankaridurg PR, et al.. Examination of the conjunctival microbiota after 8 hours of eye closure. *CLAO J*. 1995; 21(3):195-9.
- [27] Cleveland RP, Hazlett LD, Leon MA, Berk RS. Role of complement in murine corneal infection caused by *Pseudomonas aeruginosa*. *Invest Ophthalmol Vis Sci*. 1983; 24(2): 237-42.
- [28] Hazlett LD & Berk RS. Effect of C3 depletion on experimental *Pseudomonas aeruginosa* ocular infection: histopathological analysis. *Infect Immun*. 1984 Mar;43(3): 783-90.
- [29] Mondino BJ, Brown SI, Rabin BS, et al. Alternate pathway activation of complement in a *Proteus mirabilis* ulceration of the cornea. *Arch Ophthalmol* 96(9): 1659–1661.
- [30] Huang X, Du W, McClellan SA, et al. TLR4 is required for host resistance in *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci*. 2006; 47(11):4910-6.
- [31] Adhikary G, Sun Y & Pearlman E. C-Jun NH2 terminal kinase (JNK) is an essential mediator of Toll-like receptor 2-induced corneal inflammation. *J Leukoc Biol*. 2008 Apr;83(4):991-7. Epub 2008 Jan 24.

- [32] Kumar A, Zhang J & Yu F-SX. Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells. *Immunology*. 2006; 117:11–21.
- [33] Kumar A, Yin J, Zhange J, et al. Modulation of corneal epithelial innate immune response to *Pseudomonas* infection by flagellin pretreatment. *Invest Ophthalmol Vis Sci*. 2007; 48:4664–4670.
- [34] McNamara NA, Van R, Tuchin OS, et al. Ocular surface epithelia express mRNA for human beta defensin-2. *Exp Eye Res*. 1999; 69:483–490.
- [35] Maltseva IA, Fleiszig SMJ, et al. Exposure of human corneal epithelial cells to contact lenses in vitro suppresses the upregulation of human β -defensin-2 in response to antigens of *Pseudomonas aeruginosa*. *Exp Eye Res*. 2007;85:142–153.
- [36] Huang X, Du W, Barrett RP, et al. ST2 is essential for TH2 responsiveness and resistance to *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci*. 2007;48:4626–4633.
- [37] Wu X, Gao J & Ren M. Expression profiles and function of Toll-like receptors in human corneal epithelia. *Chin Med J*. 2007;120:893–897
- [38] Wu M, McClellan SA, Barrett RP, et al. Beta-defensin-2 promotes resistance against infection with *P. aeruginosa*. *J Immunol*. 2009; 182:1609–16.
- [39] Wu M, McClellan SA, Barrett RP, et al. Beta-defensins 2 and 3 together promote resistance to *Pseudomonas aeruginosa* keratitis. *J Immunol*. 2009; 183:8054–60.
- [40] Kumar A, Hazlett LD & Yu FS. Flagellin suppresses inflammatory response and enhances bacterial clearance in murine model of *Pseudomonas* keratitis. *Infect Immun*. 2008; 76:89–96.
- [41] Hume EB, Dajcs JJ, Moreau JM, et al. Staphylococcus corneal virulence in a new topical model of infection. *Invest. Ophthalmol. Vis. Sci*. 2001; 42:2904–2908.
- [42] Girgis DO, Sloop GD, Reed JM, et al. A new topical model of Staphylococcus corneal infection in the mouse *Invest. Ophthalmol. Vis. Sci*. 2003; 44:1591–1597.
- [43] Hume EB, Cole N, Khan S, et al. A Staphylococcus aureus mouse keratitis topical infection model: cytokine balance in different strains of mice. *Immunol. Cell Biol*. 2005; 83:294–300.
- [44] Bourcier T, Thomas F, Borderie V, et al. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. *Br. J. Ophthalmol*. 2003; 87:834–838.
- [45] Sloop GD, Moreau JM, Conerly LL, et al. Acute inflammation of the eyelid and cornea in Staphylococcus keratitis in the rabbit. *Invest. Ophthalmol. Vis. Sci*. 1999; 40:385–391.
- [46] Sun Y, Hise AG, Kalsow CM, et al. Staphylococcus aureus-induced corneal inflammation is dependent on Toll-like receptor 2 and myeloid differentiation factor 88. *Infect Immun*. 2006; 74:5325–32.

- [47] Huang X, Barrett RP, McClellan SA, et al. Silencing Toll-like receptor-9 in *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci*. 2005; 46:4209–4216.
- [48] Hazlett LD. Corneal response to *Pseudomonas aeruginosa* infection. *Prog Retin Eye Res*. 2004; 23:1–30.
- [49] Zepeda CJ. Resistencia de las bacterias a los antibióticos. *Revista Médica Hondureña*. 1998; 66(2): 88-92.
- [50] Henderson B, Poole S & Wilson M. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Rev*. 1996; 60(2):316-341.
- [51] Caponi G. Las poblaciones biológicas como sistemas intencionales. In: Martins, RA, Martins LA, Silva CC & Ferreira JM (eds). *Filosofia e história da ciencia no Cone Sul: 3º Encontro*. Campinas: AFHIC. 2004;212-217.
- [52] Tello A, Austin B & Telfer TC. Selective pressure of antibiotic pollution on bacteria of importance to public health. *Environ Health Perspect*. 2011 [Epub ahead of print]
- [53] Fernández-Riverón F, López Hernández J, Ponce-Martínez LM et al. Resistencia bacteriana. *Rev Cubana Med Milit* 2003; 32(1):44-48.
- [54] Van Hoek AH, Mevius D, Guerra B, et al. Acquired antibiotic resistance genes: an overview. *Front Microbiol*. 2011; 2:203.
- [55] Giedraitienė A, Vitkauskienė A, Naginienė R, et al. Antibiotic resistance mechanisms of clinically important bacteria. *Medicina (Kaunas)*. 2011; 47(3):137-146.
- [56] Roberts MC. Environmental macrolide lincosamide streptogramin and tetracycline resistant bacteria. *Front Microbiol*. 2011; 2:40:1-8.
- [57] Livermore DM. Current epidemiology and growing resistance of gram negative pathogens. *Korean J Intern Med*. 2012; 27(2):128-142.
- [58] Lim KT, Hanifah YA, Yusof M, et al. *ermA*, *ermC*, *tetM* and *tetK* are essential for erythromycin and tetracycline resistance among methicillin-resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia. *Indian J Med Microbiol*. 2012; 30(2):203-207.
- [59] Nazar CJ. Bacterial Biofilms. *Rev. Otorrinolaringol. Cir. Cabeza Cuello* 2007; 67: 61-72
- [60] Anderl JN, Franklin MJ & Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother*. 2000; 44(7):1818-1824.
- [61] Mascarenhas J, Srinivasan M, Chen M, et al. Differentiation of etiologic agents of bacterial keratitis from presentation characteristics. *Int Ophthalmol*. 2012 Jun 30. [Epub ahead of print]
- [62] Kerautret J, Raobela L & Colin J. Serious bacterial keratitis: a retrospective clinical and microbiological study. *J Fr Ophtalmol*. 2006; 29(8):883-8.

- [63] Srinivasan M, Mascarenhas J, Rajaraman R, et al. Steroids for Corneal Ulcers Trial Group. The steroids for corneal ulcers trial: study design and baseline characteristics. *Arch Ophthalmol*. 2012; 130(2):151-7.
- [64] Bourcier T, Thomas F, Borderie V, et al. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. *Br J Ophthalmol*. 2003; 87(7):834-8.
- [65] Weiss K, Ardjomand N & El-Shabrawi Y. Mycotic infections of the eye. *Wien Med Wochenschr*. 2007; 157(19-20):517-21.
- [66] Talukder AK, Halder SK, Sultana Z, et al. Epidemiology and outcome of non viral keratitis. *Mymensingh Med J*. 2011; 20(3):356-61.
- [67] Sy A, Srinivasan M, Mascarenhas J, et al. *Pseudomonas aeruginosa* keratitis: outcomes and response to corticosteroid treatment. *Invest Ophthalmol Vis Sci*. 2012; 53(1):267-72.
- [68] Lalitha P, Srinivasan M, Manikandan P, et al. Relationship of in vitro susceptibility to moxifloxacin and in vivo clinical outcome in bacterial keratitis. *Clin Infect Dis*. 2012; 54(10):1381-7.
- [69] Srinivasan M, Mascarenhas J, Rajaraman R, et al. Steroids for Corneal Ulcers Trial Group. Corticosteroids for bacterial keratitis: the Steroids for Corneal Ulcers Trial (SCUT). *Arch Ophthalmol*. 2012; 130(2):143-50.
- [70] Blair J, Hodge W, Al-Ghamdi S, et al. Comparison of antibiotic-only and antibiotic-steroid combination treatment in corneal ulcer patients: double-blinded randomized clinical trial. *Can J Ophthalmol*. 2011; 46(1):40-5.
- [71] Guyomarch J, van Nuoï DN, Beral L, et al. Infectious keratitis and cosmetic lenses: a five-case retrospective study. *J Fr Ophtalmol*. 2010; 33(4):258-62.
- [72] Donshik PC, Suchecki JK, Ehlers WH. Peripheral corneal infiltrates associated with contact lens wear. *Trans Am Ophthalmol Soc*. 1995; 93:49-60; discussion 60-4.
- [73] Mascarenhas J, Srinivasan M, Chen M, et al. Differentiation of etiologic agents of bacterial keratitis from presentation characteristics. *Int Ophthalmol*. 2012 Jun 30. [Epub ahead of print].
- [74] de Rojas V, Llovet F, Martínez M, et al. Infectious keratitis in 18,651 laser surface ablation procedures. *J Cataract Refract Surg*. 2011; 37(10):1822-31.
- [75] Suzuki T. A new target for *Staphylococcus aureus* associated with keratitis. *Cornea*. 2011; 30 Suppl 1:S34-40.
- [76] Stewart RM, Wiehlmann L, Ashelford KE, et al. Genetic characterization indicates that a specific subpopulation of *Pseudomonas aeruginosa* is associated with keratitis infections. *J Clin Microbiol*. 2011; 49(3):993-1003.

- [77] Galperín GJ, Boscaro G, Tau J, et al. Crystalline keratopathy: an infrequent corneal infection produced by the *Streptococcus mitis* group. *Rev Argent Microbiol.* 2011; 43(3):195-7.
- [78] Millender TW, Reller LB, Meekins LC, et al. Streptococcal pharyngitis leading to corneal ulceration. *Ocul Immunol Inflamm.* 2012; 20(2):143-4.
- [79] Norcross EW, Sanders ME, Moore QC 3rd, et al. Pathogenesis of A Clinical Ocular Strain of *Streptococcus pneumoniae* and the Interaction of Pneumolysin with Corneal Cells. *J Bacteriol Parasitol.* 2011; 2(2):108.
- [80] Ramos-Esteban JC, Servat JJ, Tauber S, Bia F. *Bacillus megaterium* delayed onset lamellar keratitis after LASIK. *J Refract Surg.* 2006; 22(3):309-12.
- [81] Pinna A, Sechi LA, Zanetti S, et al. *Bacillus cereus* keratitis associated with contact lens wear. *Ophthalmology.* 2001; 108(10):1830-4.
- [82] Choudhuri KK, Sharma S, Garg P, et al. Clinical and microbiological profile of *Bacillus* keratitis. *Cornea.* 2000; 19(3):301-6.
- [83] Fukumoto A, Sotozono C, Hieda O, Kinoshita S. Infectious keratitis caused by fluoroquinolone-resistant *Corynebacterium*. *Jpn J Ophthalmol.* 2011; 55(5):579-80.
- [84] Willcox M, Sharma S, Naduvilath TJ, et al. External ocular surface and lens microbiota in contact lens wearers with corneal infiltrates during extended wear of hydrogel lenses. *Eye Contact Lens.* 2011; 37(2):90-5.
- [85] Garg P, Chaurasia S, Vaddavalli PK, et al. Microbial keratitis after LASIK. *J Refract Surg.* 2010 Mar;26(3):209-16.
- [86] Suzuki T, Iihara H, Uno T, et al. Suture-related keratitis caused by *Corynebacterium macginleyi*. *J Clin Microbiol.* 2007; 45(11):3833-6.
- [87] Tay E, Rajan M & Tuft S. *Listeria monocytogenes* sclerokeratitis: a case report and literature review. *Cornea.* 2008; 27(8):947-9.
- [88] Altaie R, Fahy GT, Cormican M. Failure of *Listeria monocytogenes* keratitis to respond to topical ofloxacin. *Cornea.* 2006;25(7):849-50.
- [89] Zaidman GW, Coudron P & Piro J. *Listeria monocytogenes* keratitis. *Am J Ophthalmol.* 1990; 109(3):334-9.
- [90] Ovodenko B, Seedor JA, Ritterband DC, et al. The prevalence and pathogenicity of *Propionibacterium acnes* keratitis. *Cornea.* 2009; 28(1):36-9.
- [91] Brook I. Ocular infections due to anaerobic bacteria in children. *J Pediatr Ophthalmol Strabismus.* 2008; 45(2):78-84.
- [92] Garg P, Sharma S & Underdahl J. *Propionibacterium acnes* as a cause of visually significant corneal ulcers. *Cornea.* 200; 20(4):437-8.

- [93] Underdahl JP, Florakis GJ, Braunstein RE, et al. *Propionibacterium acnes* as a cause of visually significant corneal ulcers. *Cornea*. 2000 Jul;19(4):451-4.

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