Histopathological Studies on Rabbits Infected by Bacteria Causing Infectious Keratitis in Human through Eye Inoculation

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Abstract

Aim: This study aimed to investigate the pathogenic effect of bacteria causing infectious keratitis among patients through experimental study conducted on rabbits' eyes with the aid of histopathology as eye infection is a common disease in developing countries that may complicate to loss of vision.

Methodology: 100 swab samples were collected from human infected eyes, at Qassim region during 2012, for the isolation of *Pseudomonas aeruginosa* and *Staphylococcus aureus.* The isolated pathogenic bacteria were tested to various antibiotics using some selected antibiotics discs through agar-well diffusion method. Then, experimental study conducted on 27 rabbits. The rabbits were divided randomly into three equal groups, each containing 9 rabbits. Rabbits of group (1) served as control group (Negative Control) and their eyes were inoculated with the buffer only. Rabbits of group (2) were inoculated through eyes with the isolated *Pseudomonas aeruginosa*. Rabbits of group (3) were inoculated through eyes with the isolated *Staphylococcus aureus*.

Results: Out of 100 collected swab samples from human infected eyes, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were isolated with a total percentage of 25.21% and 15.65%; respectively and used in this study. Both bacterial isolates were sensitive to Gentamicin and Cefuroxime.

Clinically, experimentally infected rabbits by *Pseudomonas aeruginosa,* revealed varying degree corneal abrasions, corneal abscess and dense corneal opacity. Histopathologically, at 3rd day post-infection (PI), the cornea revealed polymorpho-nuclear cells infiltration with loss of the outer epithelial lining. At 7th day PI, neutrophils were seen in the stroma. At 15th day PI, proliferation of fibroblasts and new vascularisation were seen in the stroma.
Clinically, rabbits experimentally infected with *Staphylococcus aureus*, revealed corneal ulcers and focal abscesses. Histopathologically, at 3rd and 7th day PI, the cornea revealed edema and infiltration of leukocytes. At 15th day PI, hyperplasia of corneal epithelium and proliferation of keratocytes were evident. The liver and kidneys of experimented rabbits revealed no remarkable histopathological alterations along the period of experiment.

Conclusion: *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common eye infection in human, both induced severe lesions in the eyes of rabbits that could interfere with vision, therefore, strict measures to control these infections in human is recommended.

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Introduction

The cornea in domestic animals including rabbits is composed of five histological layers: anterior epithelium, subepithelial basement membrane, substantia propria or stroma, posterior limiting membrane (Descemet's membrane), and posterior epithelium (corneal endothelium). [1, 2, 3, 4] The epithelium is completely avascular and is nourished by lacrimal secretion, as well as by aqueous humor of the anterior chamber. The epithelium is characterized by a remarkable capacity for rapid repair in case of injury. [5] The sensitivity of the cornea is due to the fact that great numbers of free nerve endings are found in this layer. An intact corneal epithelium is necessary for maintenance of its transparency. [1, 2, 3]

Under ideal conditions, there is little or no opportunistic bacterial colonization of the conjunctiva or cornea, because of the washing effect of the tears, [6, 7, 8] in conjunction with the action of antibacterial proteins and enzymes within the tear film. [9, 10, 11, 12, 13] Some eye disorders have been associated with several Gram-positive and -negative bacteria, including *Staphylococcus aureus*, *Streptococcus* sp., *Bacillus subtilis*, *Rhodococcus* sp., *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Haemophilus aegyptius*, and *Klebsiella* sp. [14, 15] The production of lipases and toxins by many of these colonizing bacteria may induce ocular surface cellular damage and destabilization of the lipid layer of the tear film contributing to tear film instability, inflammation, and symptoms of significant ocular irritation. [16]

Pseudomonas aeruginosa is usually the most common bacterial pathogen isolated from cases of corneal keratitis. [17, 18, 19, 20, 210] Infection poses a serious threat to normal vision and is associated with extended wear of contact lenses, eye trauma, [22] eye surgery, and orthokeratology, [23, 24] as well as severe burns, ocular irradiation, tracheostomy, exposure to the intensive care environment, or coma. [25] Infection can occur following relative minor injury or compromise to the corneal surface and progresses rapidly, with the potential to involve the entire cornea within 2 days. The epithelial injury becomes rapidly necrotic, the underlying stroma becomes edematous, and a mucopurulent discharge develops. More severe eye involvement can then ensue, leading to perforation of the cornea, infection and inflammation in the anterior chamber of the eye, and potentially endophthalmitis. [25]

Staphylococcus aureus is the leading cause of bacterial keratitis in adults, including those who have sustained penetrating corneal injuries or are compromised by immunodeficiency. [26, 27] Tissue damage during bacterial keratitis results from the action of bacterial products on ocular tissues and from the host inflammatory response to the infection. [28, 29] *Staphylococcus* keratitis can result in irreversible corneal scarring, resulting in a loss of visual acuity.

This study aimed to isolate bacterial infection (*Pseudomonas aeruginosa* and *Staphylococcus aureus)* in infectious keratitis with investigating the mechanism of action/pathogenesis of these ocular pathogenic bacteria on rabbits through experimental infection with the aid of histopathological examinations.

Materials and Methods

Bacteriological isolation and identification:

Patients were subjected directly to the emergency department or referred from peripheral basic health units, or ophthalmologists. Swab samples (100) were collected aseptically through ophthalmologists from human infected eyes, at Qassim region in King Fahd Specialist Hospital Buraidah, Qassim Province during the period between 1st January to 31st December, 2012 for the isolation of *Pseudomonas aeruginosa* and *Staphylococcus aureus.* The samples were transferred immediately to the laboratory for processing. This technique was done routinely in collected samples including culturing, subculturing and purification, isolation and identification. The collected swabs were inoculated in tryptic soya broth overnight at 37˚C, consequently the broth was inoculated onto Blood agar, Mac Conkey's agar, Mannitol salt agar, and Chocolate agar media and incubated aerobically at 37˚C for maximum up to 48 hours, inoculated Chocolate agar plates were left in anaerobic incubator at 5% CO_2. All the bacterial isolates were identified by their colony morphology, Gram staining, pigment production, relevant biochemical tests and API strips according to the manufacturer.

Antimicrobial Sensitivity test:

The isolated pathogenic bacteria were tested to various antibiotics using some selected antibiotics discs through agar-well diffusion method as recommended by the manufacturer. All bacterial isolates were tested for their antimicrobial susceptibility against Cefoxitin (30µg), Gentamicin (10µg), Ciprofloxacin (5µg), Cefuroxime (30µg), Tobramycin (10µg), chloramphenicol (30ug) and Tetracycline (30µg). All experiments were carried out in triplicate. Each isolate was spread onto the surface of Muller- Hinton agar with a sterile swab. After 24 h of incubation, inhibition zones were measured. Control wells were filled with 50 ml. of potassium phosphate buffer of pH 7. The results of susceptibility were recorded as *Sensitive(S), Intermediate (I)* or *Resistant(R)*.

Experimental study:

This experimental study conducted on 27 rabbits. The rabbits were obtained from markets at Qassim of same size and age. The rabbits were acclimatized for 7 days in the animal house conditions before starting the experiment. The animals were weighing between 2 and 2.5kg. Rabbits were examined clinically to prove their health status (Healthy rabbits should be bright with colourful fur, neither eye discharge nor dullness or scratching are evident, their noses are twitching regularly and should not be runny), Rabbits were also subjected to bacteriological examinations to make sure they are free from infection. They were housed in standard aluminum cages and fed with standard rabbit diet and normal tap water and examined to be free from infection. They were handled as per the international rules implemented in the experimental laboratory animals, Qassim University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The Rabbits were divided randomly into three equal groups, each containing 9 rabbits; Rabbits of group (1) served as control group (Negative Control) and their eyes were inoculated with the buffer only. Rabbits of group (2) were inoculated through eyes with the isolated *Pseudomonas aeruginosa*. Rabbits of group (3) were inoculated through eyes with the isolated *Staphylococcus aureus*.

The bacterial inoculum were propagated on Mueller-Hinton agar plates and incubated at 37°C for 18 hours. Several bacterial colonies were pooled and suspended in saline to adjust the turbidity to 0.5 McFarland units (equivalent to 5-10^8 colony-forming units [CFU]/mL). The suspension was then adjusted to a final concentration of 10^5 CFU/mL, as verified by a quantitative bacterial count on Mueller-Hinton agar plates.

Rabbits were anesthetized by subcutaneous injection of a mixture of xylazine (100 mg/mL; Butler Company, Columbus, OH) and ketamine hydrochloride (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA). Proparacaine hydrochloride was topically applied to each eye. 0.05 mL (10^5 CFU/mL) from each of freshly prepared bacteria (*Pseudomonas aeruginosa or Staphylococcus aureus)* was injected into the stroma at the centre of the right cornea.

Eyes were examined macroscopically 24 hours post-infection (PI) and/or at times described below to ensure that all rabbit were similarly infected and to monitor the course of disease in infected rabbit.

Clinical examination:

Clinical examination was done to the experimented rabbits throughout the experiment using naked eye and slit lamp examination. Detailed clinical examination was covered including; visual acuity, corneal epithelial defects, epithelial erosions, corneal abrasions, number and position of corneal infiltrates, corneal abscess, hypopyon, ultimate proliferation, anterior chamber reaction, and corneal ulcer.

Histopathological techniques:

Tissue sections from the eyes and internal organs (liver & kidneys) of experimental rabbits were taken (3^{rd} ,7^{th} and 15^{th} days PI) and immediately fixed in 10% neutral buffered formalin, then dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, blocked in paraffin and sectioned as 5 µm using rotary microtome. The obtained tissue slides were stained with hematoxylin and eosin (H&E). [30]

Results:

The present study revealed the isolation of *Pseudomonas aeruginosa* (25.21%) and *Staphylococcus aureus* (15.65%) from swab samples that were collected from human Infected eyes. As shown in table (1), the isolated bacteria (*Ps. aeruginosa* and *Staph. aureus*, were sensitive to Gentamicin (10µg) and Cefuroxime (30µg).

Table (1): Antimicrobial susceptibility of the isolated bacteria using disc diffusion method.

Bacterial isolates	No	FOX	CN	CIP	CXM	TOB	C	TE
Pseudomonas aeruginosa	10	10 (R)	10 (S)	10 (S)	10 (S)	10 (S)	5 (S) 5 (I)	10 (S)
Staphylococcus aureus	7	7 (S)	7 (S)	2 (R) 5 (S)	4 S 3 (R)	3 (I) 4 (S)	2 (I) 1 (R) 4 (S)	4 (S) 2 (I) 1 (R)

FOX = Cefoxitin (30µg), CN = Gentamicin (10µg), CIP = Ciprofloxacin (5µg), CXM = Cefuroxime (30µg), TOB = Tobramycin (10µg), C = Chloramphenicol (30ug) and TE = Tetracycline (30µg).

Histopathology:

Group (1) Rabbits of control group: revealed slight ocular edema.

Group (2) Ocular infection of rabbits with *Pseudomonas aeruginosa:*

Clinical examinations of experimentally infected rabbits revealed varying degree of corneal abrasions at the site of inoculation. Corneal abscess with hypopyon and ultimate proliferation was developed within 7 days PI but full thickness dense corneal opacity leucoma was seen at 15th days PI.

Histopathologically, at 3rd day PI, the anterior layer of the stroma revealed slight neutrophils infiltration. Loss of the outer epithelial lining of the cornea (corneal ulcer) was seen (Fig. 1). At 7th day PI, marked epithelial degeneration and necrosis with subepithelial edema and congestion were evident. The stroma showed focal areas of liquefactive necrosis represented by homogenous structureless basophilic mass. The anterior and deep layer of the stroma showed loss in normal contour with loss of keratocytes (Fig. 2). At 15th day PI, edema was evident under the regenerated epithelial lining of the cornea. The stroma was intensively infiltrated with polymorpho-nuclear leukocytes (Figs. 3 & 4). Proliferation of keratocytes and new vascularisation were seen in the stroma. Loss of Descemet's membrane was commonly seen (Fig. 5).

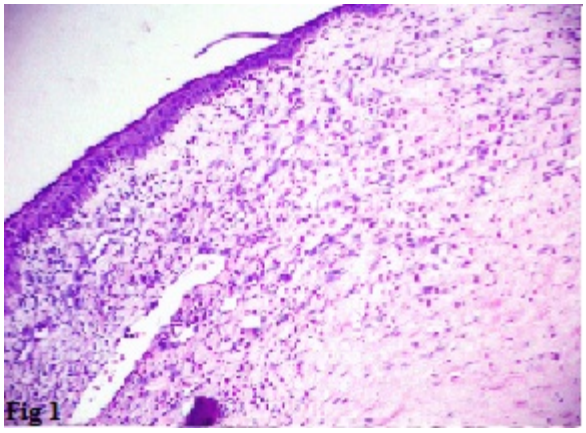

Fig. 1: Rabbit eye, infected with ***Pseudomonas aeruginosa*** at 3rd day PI, showing loss of the outer epithelial lining of the cornea and neutrophils infiltration in the anterior layer of the stroma. H & E stain x 100.

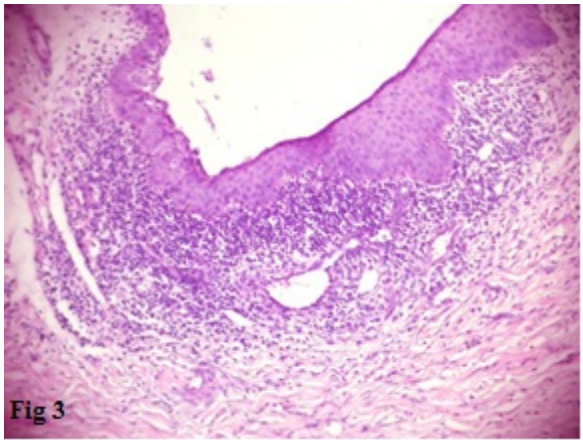

Fig. 3: Rabbit eye, infected with ***Pseudomonas aeruginosa*** at 15th day PI, showing intensive infiltration of polymorpho-nuclear leukocytes in the corneal stroma. H & E stain x 250.

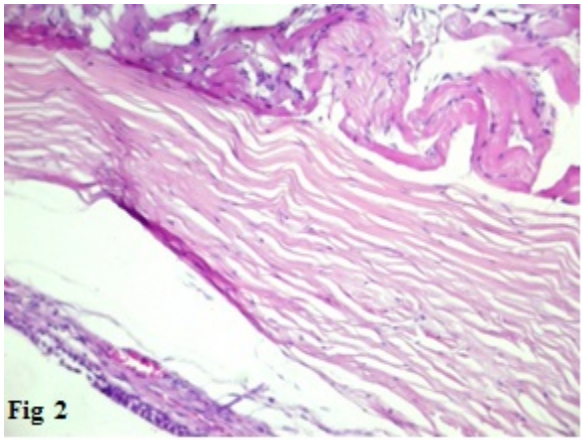

Fig. 2: Rabbit eye, infected with ***Pseudomonas aeruginosa*** at 3rd day PI, showing loss in normal contour with loss of keratocytes in the edematous stroma. H & E stain x 250.

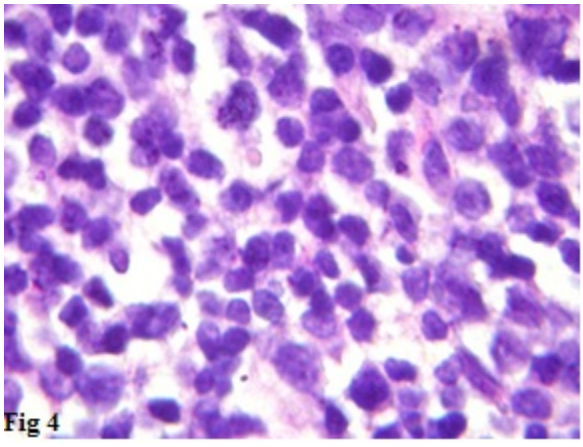

Fig. 4: Higher magnification of Fig. 3 to show the polymorpho-nuclear leukocytes. H & E stain x 1000.

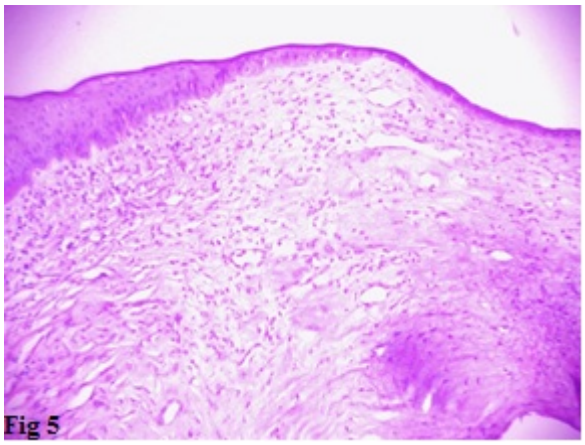

Fig. 5: Rabbit eye, infected with ***Pseudomonas aeruginosa*** at 15th day PI, showing new vascularisation and loss of Descemet's membrane. H & E stain x 250.

Group (3) Ocular infection of rabbits with *Staphylococcus aureus:*

Clinically, superficial central corneal ulcers and focal abscesses were evident in the experimental rabbits at 3rd day PI which by time healed with locomotous opacity in 15 days PI. Slit lamp examination revealed epithelial erosions and pus-filled the stromal ulcer at the injection site of the infected eyes.

Histopathologically, at 3rd day PI, the anterior and deep layers of the corneal stroma revealed intensive infiltration of neutrophils with stromal edema. Superficial central corneal epithelial ulceration was evident (Fig. 6). At 7th day PI, loss of corneal epithelium and keratocytes with central corneal vessels seen along the stroma were evident. The deeper layer of the stroma showed vascularization, edema and polymorpho-nuclear leukocytes (Fig. 7). At 15th day PI, hyperplasia of corneal epithelium was evident in un-uniform status, this in addition to proliferation of keratocytes, newly formed blood vessels and polymorpho-nuclear leukocytes infiltration in the corneal stroma (Fig. 8).

On the other hand, the histopathological examinations of the internal organs (liver and kidneys) of experimentally eye infected rabbits by either *Pseudomonas aeruginosa or Staphylococcus aureus* revealed no remarkable histopathological alterations along the period of experiment.

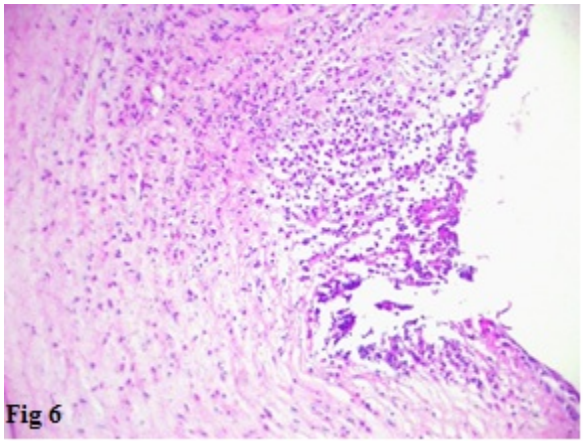

Fig. 6: Rabbit eye, infected with ***Staphylococcus aureus*** at 3rd day PI, showing corneal epithelial ulceration and intensive infiltration of neutrophils with stromal edema. H & E stain x 100.

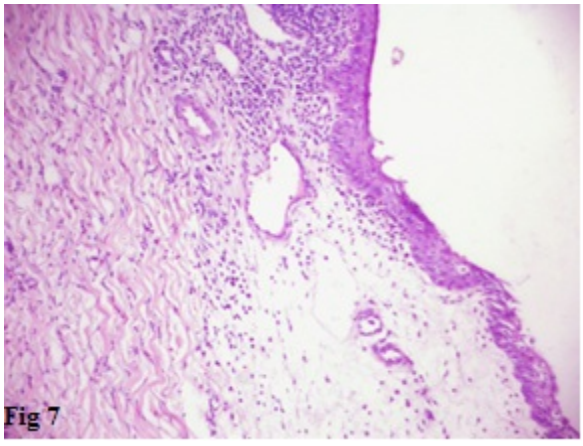

Fig. 7: Rabbit eye, infected with ***Staphylococcus aureus*** at 7th day PI, showing superficial loss of corneal epithelium and keratocytes with stromal vascularization, edema and neutrophils infiltrations. H & E stain x 100.

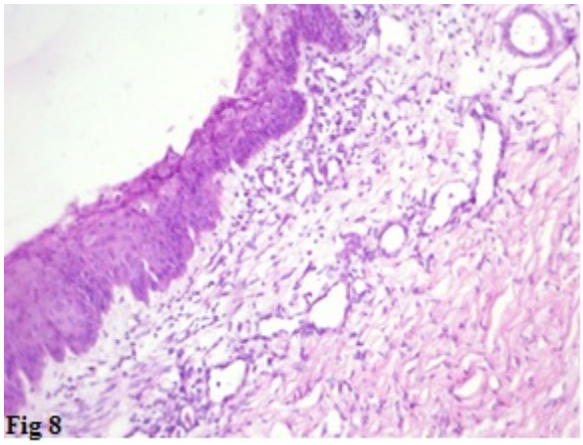

Fig. 8: Rabbit eye, infected with ***Staphylococcus aureus*** at 15th day PI, showing hyperplasia of corneal epithelium with proliferation of keratocytes, newly formed blood vessels and polymorpho-nuclear leukocytic infiltration in the corneal stroma. H & E stain x 250.

Discussion

Bacterial keratitis continues to be a sight-threatening disease despite the development of potent new antibacterial agents. In spite of intensive antibiotic treatment, corneal damage can occur as a result of keratolytic and inflammatory processes caused by infection or scarring of neovascularisation related to the healing process. [31, 32] The most predominant microbial pathogen of infectious keratitis is *Pseudomonas aeruginosa,* [33] also both of *Staphylococcus* species (*aureus* and *epidermidis*) and the *Streptococcus* species are isolated. [34] In the study carried by Ren, et al. [35] a total of 168 organisms were isolated from 728 intraocular specimens. Overall, 96 (57.1%) of 168 isolates were Gram-positive cocci, 52 (31.0%) were Gram-negative bacilli, 18 (10.7%) Gram-positive bacilli, and 2 (1.2%) Gram-negative cocci. The most common organisms identified in that study were *Staphylococcus epidermidis* in 21.4% (36/168), *Staphylococcus aureus* in 11.3% (19/168), and *Pseudomonas aeruginosa* in 8.9% (15/168). Similar results were reported also by Fong et al.,[36] who mentioned that *Pseudomonas* species were the most commonly isolated organisms (46.7%), followed by *Staphylococcus* species (11%), on the other hand Leibovitch et al.,[37] recorded that coagulase negative Staphylococcus was the commonest pathogen identified (29.8% of positive cultures), followed by *Staphylococcus aureus* (18.7%), *Pseudomonas aeruginosa* (12.7%), *Moraxella* sp. (6.7%), *Streptococcus pneumonia* (6.0%), and fungal keratitis (5.2%). The previous data encourage us to test the pathogenicity of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in the present study where both bacteria were isolated from the collected samples of the current study with a prevalence of 25.21 %, 15.65 %; respectively. The tested bacteria (*Staphylococcus aureus and Pseudomonas aeruginosa)*, were sensitive to Gentamicin (10µg) and Cefuroxime (30µg) (Table 1). Similar study stated that, *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates from corneal infection were susceptible to cefuroxime/gentamicin. [38] Other recorded revealed that, *Pseudomonas aeruginosa* isolates were susceptible to gentamicin. [39]

The role of epithelial injury in the development of corneal infection is well established. Damage to the epithelium provides adherence sites for bacterial attachment and direct access to the corneal stroma. [40] Trauma and ocular surface disease are both recognised risk factors for the development of microbial keratitis in temperate regions, accounting for approximately 50% of cases. [41,42] After corneal injury or during contact lens wear, *Pseudomonas aeruginosa* can cause sight-threatening corneal disease. [43, 44] However researchers who study corneal infection commonly use “invasive” methods to enable disease, including scarification before inoculation or intrastromal injection of the inoculums. [45, 46] It is assumed that, the corneal epithelium provides the major barrier to microbes in vivo and the scarification and intrastromal injection methods work because they enable bacteria to bypass it.

Ps. aeruginosa isolates from corneal infections have been divided into two types based on their different effects on corneal epithelial cells in vitro. [47, 48] Invasive and cytotoxic strains occur in approximately equal numbers in human corneal disease and both can cause keratitis in mice. [48,49] Invasive strains enter corneal epithelial cells and then replicate within their cytoplasm. [50, 51] Although

cytotoxic *Ps. aeruginosa* can also invade cells. [52] Our histopathological results showed slight neutrophilic infiltration in anterior layer of the stroma at 3rd day PI together with loss of the outer epithelial lining of the cornea (corneal ulcer). At 7th day PI, intense collection of neutrophils in the stroma was seen with focal areas of liquefactive necrosis represented by homogenous structureless basophilic mass. At 15th day PI, edema was evident under the regenerated epithelial lining of the cornea. The stroma was intensively infiltrated with polymorpho-nuclear leukocytes with proliferation of fibroblasts and new vascularisation.

Similar results recorded by Twining et al., [53] who stated that, when higher numbers of *Pseudomonas aeruginosa* (10^7-10^8) were applied to the scratched corneas, all corneas became ulcerated within 24 hours. Most of the corneal destruction occurred in the central epithelium and anterior stroma. Inflammatory cells were associated with all three layers, including the endothelial cell layer. The major inflammatory cells present in this area were neutrophils. It has been suggested that, this rapid corneal destruction may be the result of an extremely potent bacterial proteolytic enzyme. [54] This has been described as being intracellularly synthesized by *Pseudomonas aeruginosa*, released and extracellularly activated by available calcium cations. [55] The ability to split certain synthetic hexapeptides in a highly specific manner, plus its apparent action on the corneal stroma which is largely collagen, suggests further that, this metallo-enzyme is in fact a collagenase. The ability of a crude extract of this enzyme when injected intra-stromally to mimic an infection in the absence of bacteria suggests further that, this is the bacterial product which is primarily responsible for the extensive corneal destruction that seen in *Pseudomonas aeruginosa* keratitis. [56] Similar resulted mentioned previously. [57, 58]

Staphylococcus aureus keratitis is one of the commonest forms of bacterial keratitis. The presence of corneal infection bacterial proteins act as a chemotactic stimulus that direct the migration of neutrophils towards the area of bacterial proliferation, which is followed by an influx of macrophages. Clinically, in experimental models, marginal corneal infiltrates are the result of a T cell mediated hypersensitivity reaction to staphylococcal cell antigens that attract polymorpho-nuclear leucocytes and mononuclear cells. [59] The ring of infiltration is thought to result from complement activation caused either by an intra-corneal hypersensitivity reaction against antigens diffusing from a focal source attracting neutrophils, or complement may be activated by an antibody independent mechanism via the alternative pathway by bacterial toxin. [60] Although epithelial injury alone may produce a leucocyte chemotactic factor, [61] dense stromal infiltrates do not develop in most cases of epithelial injury. Our histopathological findings revealed edema and intensive infiltration of polymorpho-nuclear leukocytes in the eye stroma at 3rd day PI together with superficial central corneal epithelial ulceration. At 7th day PI, there were loss of keratocytes with central corneal vessels seen along the stroma. The deeper layer of the stroma showed marked congestion, edema and polymorpho-nuclear leukocytes at 15th day PI, hyperplasia of corneal epithelium and proliferation of keratocytes in the corneal stroma were seen. These findings are in accordance with O'Callaghan et al., [62] who mentioned that *Staphylococcus aureus* corneal infection results in extensive inflammation and tissue damage. Application of purified alpha-toxin produced corneal epithelial erosions and iritis, while application of beta-toxin caused scleral inflammation. These studies confirm the role of alpha-toxin as a major virulence factor during *Staphylococcus aureus* keratitis and implicate beta-toxin, a mediator of edema**,** as a lesser contributor to ocular damage. Similar resulted were previously recorded. [63, 64] Overall, although infectious keratitis is caused by many bacteria, fungi, acanthamoeba and/or viruses, and infected cases may be diagnosed/ assessed by several methods, our current study was limited to most prevalent pathogenic bacteria contributing to such infections (*P. aeruginosa* and *S. aureus*) and the diagnosis/pathogenesis was done through the unique histopathological investigations.

Conclusion:

Pseudomonas aeruginosa and *Staphylococcus aureus,* as common pathogens causing infectious keratitis in human, induced severe pathologic lesions in the eyes of rabbits upon intrastromal injection

that could interfere with vision or may cause permanent alterations. Strict measures are advised to control these infections in human and to avoid ocular impairment.

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