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Review Article

The role of Type III secretion system in the pathogenesis of *Pseudomonas aeruginosa* microbial keratitis

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Abstract

Pseudomonas aeruginosa is the most commonly isolated Gram-negative pathogen causing sight-threatening microbial keratitis (MK). Contact lens wear is the most significant risk factor associated with pseudomonal MK. Understanding the pathogenesis of MK due to *P. aeruginosa* and its interactions with contact lenses is crucial in preventing these often rapidly progressive and highly antibiotic-resistant infections. Bacterial virulence factor Type III secretion system (T3SS) has significant interplays between contact lens material, antibiotic sensitivity, disinfectant selectivity, and bacterial cell invasion. Depending on the T3SS exotoxins produced, *P. aeruginosa* strains are divided into cytotoxic or invasive strains. Cytotoxic strains are relatively resistant to commercial disinfectants, while invasive strains are more antibiotic resistant. Therefore, contact lens wearers are more predisposed to cytotoxic *P. aeruginosa* infections, and patients with trauma or previous surgery are more prone to infection by invasive strains. Previous studies with mutant *P. aeruginosa* strains unable to produce T3SS exotoxins were more susceptible to disinfectants and less able to adhere to soft contact lenses, indicating an essential role of T3SS in bacterial virulence. Invasion of *P. aeruginosa* intracellularly was found to be associated with control of scaffold protein IQ-domain GTPase-activating protein 1 (IQGAP1) and human corneal epithelial cell tight junctions. Knockdown of IQGAP1 strengthened tight junctions that prevented intracellular survival of invasive *P. aeruginosa* strains and enhanced corneal epithelial cell survival. These novel findings of the vital role of T3SS in the pathogenesis of pseudomonal MKs will provide new guidelines in both prevention and treatment of this common eye-blinding infection.

Keywords: *Contact lens, IQ-domain GTPase-activating protein 1, Microbial keratitis, Pseudomonas aeruginosa, Type III secretion system*

Introduction

Microbial keratitis (MK) due to *Pseudomonas aeruginosa* causes severe ocular morbidity that may result in blindness if not treated promptly and appropriately [1]. *P. aeruginosa*, an opportunistic pathogen commonly found in our environment, is the most commonly isolated Gram-negative organism causing MK, particularly among contact lens wearers [2]. The incidence of this contact lens-related microbial keratitis (CLMK) is approximately 3.5–20.9 per 10,000 wearers, varying due to contact lens material and wearing schedules [3-5]. With young emmetropic individuals wearing cosmetic color-tinted contact lenses, the incidence of CLMK is unfortunately expected to increase [6,7].

Ongoing research to understand the complex interaction between contact lens material, the initiation mechanisms of CLMK, and the bacterial–host immune response has continued to shed light into the perplex mechanisms causing MK. As our study and other previous studies have shown, contact lens material significantly affects the amount of bacterial adhesion [8-11]. Virulence factors of the bacterium may also influence its propensity to adhere [9,12-14]. Among the multitude of virulence factors, the Type III secretion system (T3SS), a contact-dependent protein secretion pathway secreting exotoxins, has been associated with significant host cell damage and thus clinical disease [15-17]. In this article, we review the present

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understanding of the complex interactions between *P. aeruginosa* with contact lenses material in the initiation process of the pathogenesis of CLMK.

Pathogenesis of *Pseudomonas aeruginosa* microbial keratitis

Pseudomonal keratitis often presents as a rapidly progressive stromal ulceration with overlying epithelial defect. The stromal ulceration may lead to severe and often rapid stromal melting, resulting in corneal perforation and visual loss [18,19]. The development of CLMK commences with bacterial contamination and subsequent bacterial adhesion to contact lenses, rendering prolonged exposure of the cornea to microbial pathogens [Figure 1] [20-22]. Since *P. aeruginosa* is an opportunistic organism, the establishment of an infection requires a compromised state of the host. Trauma due to abrasion or hypoxic injury due to contact lens wear had been proposed to cause epithelial breakdown and thus allow ensuing pseudomonal invasion [12,23,24]. To increase the oxygen permeability to the cornea during contact lens wear, silicone hydrogel materials were developed and were found to have at least 6 times greater oxygen permeability than conventional hydrogel lenses [25]. Nevertheless, daily wear of silicone hydrogel contact lenses still had about 6 times greater risk of corneal infection than daily wear or daily disposable conventional soft contact lens [26]. Clearly, factors other than hypoxia must be involved. Therefore, besides the contact lens material itself, the interaction of bacterial virulence factors with host defenses or immunity had been previously studied.

Pseudomonal Type III secretion system

T3SS exoenzymes were found to be important virulence factors inducing *P. aeruginosa* keratitis [27-30]. The T3SS is a specialized protein export system that forms a needle-like complex between bacterial and host cells for the transport and secretion of four exotoxins, ExoS, ExoU, ExoT, and ExoY [Figure 2] [17]. The T3SS is encoded by 36 genes in five operons, which correspond to five functional components: the needle-like complex, the translocation apparatus, the regulatory proteins, the chaperones, and the effector proteins [17]. Structurally, the needle-like complex is a hollow filament about 60–120 nm long and 6–10 nm wide and serves as a tube through which secreted factors are transported [31]. The translocation apparatus is composed of proteins that form pores on the host's cell membrane. The needle-like complex connects with these pores, which then accept the effector proteins transported by the needle-like complex and deliver it across the host's cell membrane. A structural component of the needle complex, PscC protein is located on the outer membrane of *P. aeruginosa* and has been shown to be essential in effector transport [Figure 2] [17,32]. Mutations in the PscC protein result in loss of bacterial virulence and also T3SS exotoxin secretion [32-34]. The expression of the T3SS usually requires close cell contact *in vivo* or low calcium growth conditions *in vitro* [35-37]. Contamination of contact lenses with adhering bacteria thus provides a chance for relatively close contact with the corneal epithelium to induce the T3SS *in vivo*. After T3SS activation, the chaperone proteins which bind to effector proteins before secretion facilitate the delivery of effector proteins into the secretion system [38]. Chaperones remain in the bacterial cytoplasm after their effector protein

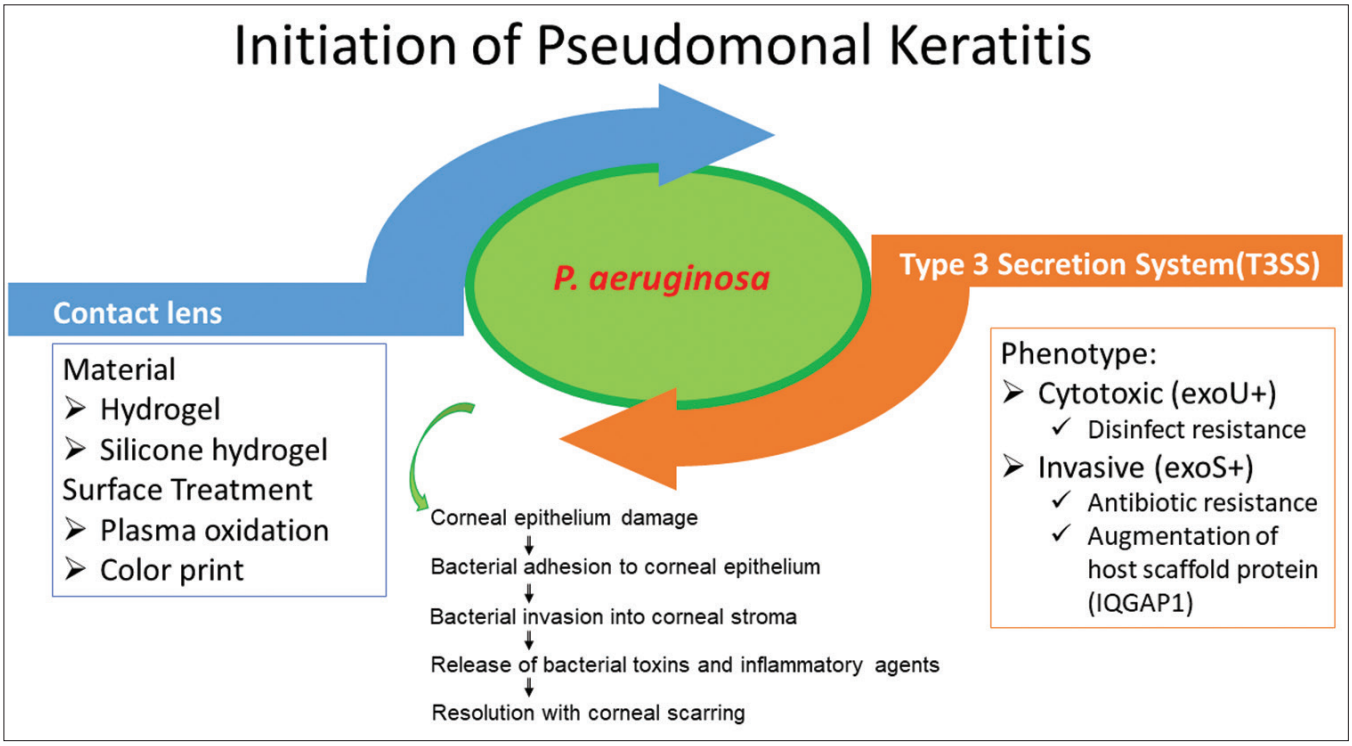

Figure 1: Proposed pathogenesis of pseudomonal keratitis. Complex interaction between bacterial virulence factor Type III secretion system, contact lens, and host immune response occurs during the initiation process of *Pseudomonas aeruginosa* keratitis. Understanding of these complex interactions may help to develop prevention strategies and target therapy

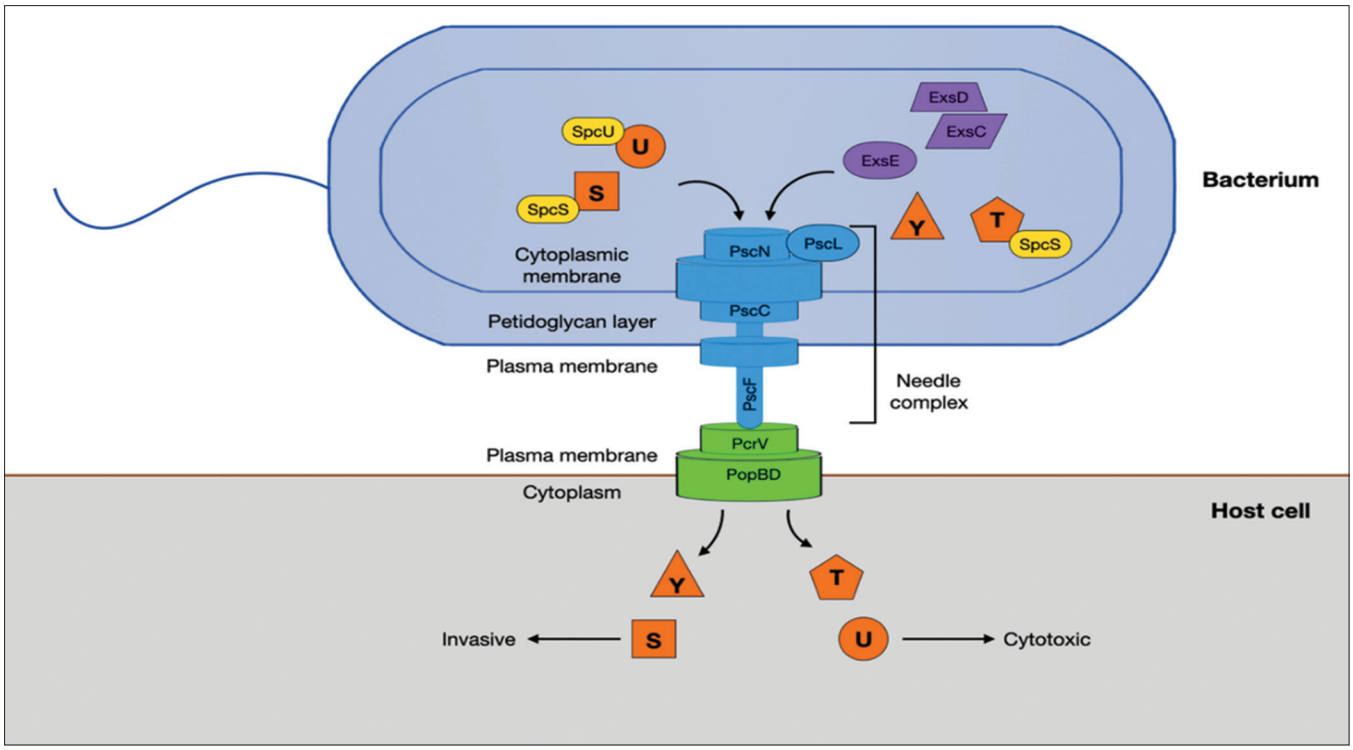

Figure 2: The Type III secretion system of *Pseudomonas aeruginosa* [17]. Type III secretion system is a specialized contact-dependent or low calcium-induced bacterial virulence system. Transport of exotoxins through needle-like complex affects host cells either by acute cell lysis (ExoU) or host invasion (ExoS)

partners have been secreted. The most important component of the T3SS is the effector proteins, which are the "payload" that is injected into the host cell that disrupts various cellular processes, leading to clinical disease.

Currently, only four effector proteins of the *P. aeruginosa* T3SS have ever been identified despite extensive characterization: ExoS, ExoU, ExoT, and ExoY.

ExoS is a 453-amino acid (48 kDa) cytotoxin that exhibits both GTPase-activating protein (GAP) activity and ADP ribosyl transferase (ADPRT) activity [39]. Analysis of the protein sequence suggests that residues 96–233 contain the GAP domain, which targets small GTPases such as Rho, Rac, and cell division cycle 42 (CDC42) and turns them into an inactive form, disrupting host cell actin cytoskeleton organization. Residues 233–453 contain the ADPRT domain, with residues 418–429 containing a binding site for a 14-3-3 protein necessary for the activation of ADPRT activity [40]. The requirement for a 14-3-3 protein – a eukaryotic cofactor – protects the bacteria against self-damage until ExoS is secreted into the host. Once injected, ADPRT activity of ExoS becomes active and disrupts the host's actin cytoskeleton, which then interferes with vesicular trafficking and endocytosis, ultimately leading to cell death with the features of apoptosis or necrosis [41]. The disruption of the host's cytoskeleton can also reduce cell–cell adherence, facilitating *P. aeruginosa* invasion through epithelial barriers [17].

In contrast to ExoS, ExoU (74 kDa) is a phospholipase that causes host cell lysis within 1–2 h [42]. Analysis of its protein sequence reveals a patatin-like domain with phospholipase A_2 (PLA_2) activity between residues 107 and 357 [43]. PLA_2 enzymes hydrolyze the ester bond of phospholipids at the S_N2 position, resulting in the release of free fatty acids and lysophospholipids [43,44]. To facilitate host cell lysis, ExoU contains a membrane localization domain between residues 550–687 that directs ExoU to the phospholipid plasma membrane of the host cell [45]. To ensure tha this potent weapon is not turned against the bacteria itself, ExoU requires activation by a eukaryotic cofactor: Cu^{2+}, Zn^{2+}-superoxide dismutase 1 [43,46]. Taken altogether, ExoU may be used to kill the host's epithelial cells and immune cells, thereby promoting bacterial invasion and dissemination [17].

ExoT is a 457-amino acid (40 kDa) cytotoxin that shares 76% homology with ExoS [47]. Similar to ExoS, residues 78–235 of ExoT contain GAP activity toward Rho, Rac, and CDC42, causing reversible disruption of the actin cytoskeleton. This leads to cell rounding, cell detachment, and inhibition of cell migration, phagocytosis, and cytokinesis [48,49]. Further, like ExoS, residues 235–457 of ExoT contain an ADPRT domain that similarly requires binding of the host cell 14-3-3 cofactor for activation. While ExoT ADP ribosylates a different set of host proteins [50], the net effects of GAP and ADPRT activity in ExoT are similar to ExoS, which is actin cytoskeleton disruption and inhibition of cell adhesion and phagocytosis. This allows *P. aeruginosa* to disseminate by evading phagocytosis and breaking down epithelial barriers [17,49].

ExoY is a 378-amino acid (42 kDa) adenylyl cyclase that also requires a host cell cofactor for activation, although the identity of this cofactor is currently unknown [51]. When ExoY was injected into mammalian cells, elevated intracellular cAMP concentrations and differential gene expression were

observed, which led to the disruption of the actin cytoskeleton, inhibition of host phagocytosis of bacteria, and increased endothelial permeability [17,51].

For reasons that are unclear, most strains do not carry all four genes encoding for the four effectors. In fact, while almost all strains of *P. aeruginosa* carry both *exoT* and *exoY* genes, most have only either *exoU* or *exoS*, not both [17]. As such, two genotypes of *P. aeruginosa* with different infection phenotypes can be distinguished by the secretion of either ExoU or ExoS. Strains that are ExoS positive and ExoU negative are known as invasive strains as they can invade into corneal epithelial cells [27,52-54]. On the other hand, strains that are ExoU positive and ExoS negative are known as cytotoxic strains because ExoU, a phospholipase, causes acute host cell lysis [53,55]. The invasive and cytotoxic phenotypes with their respective *exoS* and *exoU* genotypes were found to be mutually exclusive in nearly all strains [27].

Pseudomonal Type III secretion system and clinical manifestation of microbial keratitis

From our investigation of invasive and cytotoxic strains isolated from MK cases over a 10-year period in Taiwan, the two phenotypes of *P. aeruginosa* showed significant differences both in clinical manifestation and in visual prognosis. Clinical *P. aeruginosa* isolates were mostly of cytotoxic strains among contact lens wearer, while invasive strains were significantly related to a history of trauma or previous surgery [9,56]. This was similarly noted among Australian isolates [57]. Cytotoxic strains were also more commonly seen in young females in contrast to invasive strains, which predominantly occurred in older males [56]. Initial presenting visual acuity for cytotoxic strains was also significantly better compared to MK due to invasive strains. This is mainly due to smaller infiltration size and less fulminant disease due to cytotoxic strains [56]. Invasive strains often had a greater infiltration size and depth accompanied by the presence of hypopyon therefore poorer final visual outcome and even higher treatment failure rate (Failure was defined as severe uncontrollable infection requiring therapeutic penetrating keratoplasty, evisceration, or enucleation) [56].

Antibiotic susceptibility varied according to the regions and between the two phenotypes. A greater proportion of cytotoxic strains from the USA, Australia, and India were more resistant to fluoroquinolones and aminoglycosides, although differences between phenotypes were not significant [58-60]. This is in contrast to isolates from Taiwan, which showed an increased antibiotic resistance to fluoroquinolones for invasive strains [56]. Cytotoxic strains from CLMK cases were all sensitive to commonly used aminoglycosides and fluoroquinolones [56]. These results suggest that analysis of *P. aeruginosa* phenotype may be helpful clinically in predicting visual prognosis and also suggesting clinical guidelines for the treatment of pseudomonal keratitis with some regional differences.

Type III secretion system and host invasion

The effect of T3SS effectors on corneal epithelial cells differ with their genotypes. Strains with the *exoS* genotype can invade and multiply within corneal epithelial cells both *in vitro* and *in vivo* [52,55]. It was found that functional T3SS was required for intracellular survival of *P. aeruginosa* within corneal epithelial cells [52]. Wild-type strains with functional T3SS can be seen replicating within plasma membrane blebs [61]. In contrast, the T3SS needle-complex *pscC* mutant could not form membrane blebs and thus had reduced intracellular survival [61]. Thus, these membrane blebs are essentially used as niches by *P. aeruginosa* for intracellular survival and replication [61]. Visualization of the membrane localization of intracellular *P. aeruginosa* can be seen with confocal microscopy using GFP-transformed strains [62]. Although preliminary work on how ExoS promotes intracellular survival had been done, the studies of ExoS on disruption of epithelial barrier function were mostly done on mucosal cells of the lung [63].

The effect of ExoS with a scaffold protein IQ-domain GTPase-activating protein 1 (IQGAP1) on the corneal epithelial barrier was recently published by ShenW *et al*. [64]. IQGAP1 co-localized with junctional proteins actin and zonular occludin 1 to induce changes in the corneal epithelial cell tight junction [64]. Knockdown of IQGAP1 enhanced tight junction formation with increased transepithelial resistance [64]. Since invasive strain *P. aeruginosa* invades human corneal epithelial cells by rapidly breaking down tight junctions, enhancement of tight junctions resulting from the knockdown of IQGAP1 can protect against *P. aeruginosa* invasion, leading to enhanced cell survival [64]. Viability of cells increased by 45% early in the infection [64]. After IQGAP1 knockdown, PAK was also less able to invade and survive within the corneal epithelial cells [64]. These novel findings suggested that T3SS enables invasiveness of *P. aeruginosa* through breakdown of tight junctions. Invasion of the bacterium into host cells protected the pathogen from host immune defenses and evaded antibiotics control. The poor clinical outcomes due to invasive pseudomonal strains may be partially due to this evasion tactic. Therefore, understanding the mechanism of pseudomonal invasion is essential in the development of new therapeutic targets, especially with rising antibiotic resistance.

Type III secretion system and contact lenses

The T3SS virulence factors not only influence clinical prognosis and bacterial antibiotic sensitivity, but it has also been shown to affect pseudomonal adherence to contact lenses. Previously, environmental and clinical isolates from lung, urinary tract, or burn wound infections were shown to be mostly invasive [65-67]. However, the higher prevalence or predisposition of finding cytotoxic genotype among contact lens wearers suggested that T3SS may be involved either directly or indirectly with adhesion to contact lens materials. By using low calcium-inducing conditions to compare the bacterial adhesions between wild-type and *pscC*-mutant strains, it was demonstrated that *pscC* mutants were unable to secrete T3SS exotoxins, irrespective of strain. These mutants showed significantly less adhesion to both conventional hydrogel and silicone hydrogel contact lenses compared to wild-type strains [9]. Compared to noninducing conditions, bacteria grown under T3SS-inducing

conditions also showed significantly greater bacterial adhesion to contact lenses, indicating that functional and active T3SS is essential for contact lens adhesion [9]. Although functional T3SS affects bacterial lens adhesion, genotype differences were not found. Therefore, other factors must be involved in the predisposition of cytotoxic genotype among CLMK.

Contact lens material had been shown to affect pseudomonal adherence. Regardless of strain, silicone hydrogels with plasma oxidation surface treatment tend to adhere greater bacteria [9]. Generally, galyfilcon silicone hydrogel lenses (lenses without surface treatment) had the least amount of bacterial adhesion compared to surface-treated silicone hydrogel and conventional hydrogels [9]. Thus, surface smoothness was proposed to affect bacterial adhesion. Cosmetic hydrogel lenses that easily dislodge color pigments were shown to adhere significantly more bacteria [68,69]. Scanning electron microscope of pigmented contact lenses showed a significant association of surface smoothness with pigment dislodgement [70]. Therefore, improper contact lens material or surface treatment may increase the propensity for bacterial adhesion onto lens surfaces.

Type III secretion system and disinfectant selectivity

As previously mentioned, functional T3SS was required for bacterial adherence to contact lenses. However, there were no genotype differences in adhesion demonstrated by us and others [9,71]. Selectivity of cytotoxic strains by disinfectants or multipurpose solutions was thought to be a possible explanation. In 2001, Lakkis and Fleiszig found an increase in resistance to two contact lens chemical disinfectants [72]. This resistance was associated with acute cytotoxic activity to corneal epithelial cells [72]. Recently, we compared the sensitivity of the two genotypes of *P. aeruginosa* to four commercially available disinfectants (Renu Fresh, PureMoist, Replenish, and AoSept Plus). Cytotoxic strains were significantly more resistant to disinfectants, especially Renu Fresh, even at manufacturer's recommended disinfection time. The best disinfectant was AoSopt Plus, the hydrogen peroxide system, which was able to completely eradicate *P. aeruginosa* even at 25% of recommended disinfection time. Compared to standard wild-type strains, *pscC*-mutant strains were all susceptible to disinfectants, indicating that functional T3SS may contribute to bacterial disinfectant resistance. These cumulative findings suggest that the relative higher prevalence of cytotoxic strains with contact lens wearers may be due to the relative selectivity of marketed disinfectants. Careful selection of effective disinfectants should be advocated for proper lens hygiene.

Conclusion

P. aeruginosa is the most common pathogen causing vision-threating corneal infections, especially in contact lens wearers. The rapid progressive course and poor clinical outcomes due to emerging antibiotic resistance stimulated extensive research in the role of bacterial virulence factor T3SS interactions with contact lenses. As extensive reviewed, recognition of the importance of the various effects of T3SS exotoxins will better enable early prevention measures and novel treatment options to salvage the devastating sight-threatening complication due to *P. aeruginosa* infections.

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Conflicts of interest

There are no conflicts of interest.

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