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Pseudomonas aeruginosa and microbial keratitis

Yasmin Hilliam[1], Stephen Kaye[2] and Craig Winstanley[1,*]

Abstract

Pseudomonas aeruginosa, a versatile Gram-negative pathogen that can cause a wide range of infections, is the most common causative agent in cases of bacterial keratitis associated with contact-lens use. Corneal infections with *P. aeruginosa* often have poor clinical outcomes and can result in long and costly treatments. During the infection process, the pathogen exploits its large genome, encoding complex regulatory networks and a wide range of virulence factors, including motility and the secretion of various proteases and toxins. Although antibiotic resistance levels in the UK are low, higher levels have been seen in some other countries. In the face of increasing antibiotic resistance, alternative therapeutic approaches such as antivirulence strategies and phage therapy are being developed. There is increasing evidence to suggest that keratitis infections are associated with a phylogenetic subgroup of *P. aeruginosa* isolates carrying the gene encoding the potent cytotoxin exotoxin U, one of two mutually exclusive exotoxins secreted via the type III secretion system. The mechanisms behind this association are unclear, but understanding the genetic differences that predispose *P. aeruginosa* to cause corneal infections may allow for the development of targeted and more effective future treatments to reduce the morbidity of *P. aeruginosa* keratitis. In order to minimize the risk of severe *P. aeruginosa* eye infections, a wide range of contact-lens disinfection solutions are available. Constant exposure to biocides at a range of concentrations, from sub-inhibitory to inhibitory, could contribute to the development of resistance to both antibiotics and disinfectants.

MICROBIAL KERATITIS

Keratitis is an ophthalmological disease in which the cornea becomes inflamed and it can result in partial or total loss of vision in severe cases. The disease can result from microbial infection (e.g. bacteria, fungi, viruses or protozoans) or from non-infectious damage, such as that caused by eye trauma or exposure to chemicals or ultraviolet light [1]. Microbial keratitis, however, usually involves ulceration of the cornea with subsequent neovascularization, scarring and loss of vision. The aetiology and occurrence of microbial keratitis varies geographically, depending largely on climate and level of industrialization. After cataracts, bacterial keratitis is the second-largest cause of legal blindness worldwide [2]. Keratitis also incurs significant annual costs to healthcare providers around the world. Data from the USA estimated that 988 000 visits were made to doctors' surgeries, outpatient clinics and hospital emergency departments in 2010 for keratitis and contact-lens-related illnesses. In total, these visits were estimated to cost $174.9 million and over 250 000 h of clinician time [1]. Quantification of the costs incurred by admission and treatment of keratitis patients by the National Health Service (NHS) in the UK was carried out over a period of 12 months (January–December 2013) using data collected from 101 patients with microbial keratitis attending a tertiary referral centre. The total calculated cost of admission for all patients was £382 473, with the total income generated calculated as £267 028, giving a deficit of £115445 per annum for this centre alone [3]. Length of stay was determined to be the most critical factor in increasing the cost deficit, with surgical intervention also driving up the cost of care.

In cases of bacterial keratitis, the most common causative agents are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, coagulase negative *Staphylococcus*, *Streptococcus* spp. and *Enterobacteriaceae* [4]. In cases associated with contact-lens use *P. aeruginosa* is consistently the most commonly isolated microbe from corneal scrapings [5–7].

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Author affiliations: [1]Department of Clinical Infection, Microbiology, and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool, L69 7BE, UK; [2]Department of Eye and Vision Science, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, L7 8TX, UK.
***Correspondence:** Craig Winstanley, c.winstanley@liverpool.ac.uk

Abbreviations: ADP, Adenosine diphosphate; cAMP, 3',5'-yclic adenosine monophosphate; EDTA, Ethylenediaminetetraacetic acid; G + C, Guanine-cytosine; GTP, Guanosine-5'-triphosphate; IL, Interleukin; IQS, Integrating quorum sensing signal; MIC, Minimum inhibitory concentration; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PQS, Pseudomonas quinolone signal; T3SS, Type III secretion system.

Contact-lens-associated microbial keratitis

For more than 30 years, the use of contact lenses has been acknowledged as a predisposing factor in the development of microbial keratitis [8], with the use of contact lenses increasing at an annual rate of 5–15% [9]. The contribution of individual factors, however, including lens material, duration of use, cleaning and hygiene, and lens quality must also be taken into consideration. Most recent figures estimate worldwide contact-lens use at 140 million patients, with 10–12% of the population in the USA and UK using contact lenses regularly for vision correction [9]. Annually, contact-lens-associated microbial keratitis is estimated to affect 1 in 2500 'daily use' patients and 1 in 500 'overnight use' patients [10–13]. Whilst it is not the only causative agent of bacterial keratitis associated with contact-lens use, *P. aeruginosa* is responsible for the majority of corneal ulcers associated with lens wear [8, 14].

P. AERUGINOSA

P. aeruginosa is a versatile Gram-negative bacterium that can occupy a wide range of environmental niches but also acts as an opportunistic pathogen, causing numerous different kinds of infection [15]. *P. aeruginosa* is an important pathogen in worldwide healthcare and is included in the ESKAPE pathogen group commonly associated with concerning increases in antimicrobial resistance (AMR) [16]. It has also recently been identified by the World Health Organization as one of the top three priority 1 pathogens urgently requiring development of new antibiotics for effective treatment of infection [17]. *P. aeruginosa* is responsible for approximately 10% of nosocomial infections in the European Union [18]. It frequently causes infections in severely immunocompromised individuals (such as cancer patients) or neonates, people with severe burns or wounds, patients subjected to invasive procedures (such as the use of catheters or ventilators) or individuals with debilitating disorders such as cystic fibrosis. It is also the leading cause of sight-threatening corneal disease in otherwise healthy patients who use contact lenses [19, 20]. The versatility of *P. aeruginosa* and its ability to cause a wide range of human diseases is attributed to its large genome [21], which confers metabolic flexibility and adaptability.

Variation in the pathogenicity of *P. aeruginosa* is driven largely by diversity among the global population. Not only is the *P. aeruginosa* genome large (generally >6 Mbp), but it is composed of a core genome of well-conserved genes carried by all strains interspersed with regions of genomic plasticity (RGP) at specific sites on the genome, which contain accessory genes [22]. These accessory genome regions can vary significantly between strains and contribute to pathogenicity. A recent study using a dataset from 1311 strains reported a pangenome (total genomic content of the population) of 54272 genes, with only 665 representing the strict core genome (present in all strains). However, 26420 of these genes were assigned to the 'flexible genome' (present in some strains but not others), with 27187 being unique (present in only one strain) [23]. The *P. aeruginosa* genome has a high G+C content (approximately 67%), with genes acquired from other species, often found as part of the accessory genome, having a notably lower G+C content. Phylogenetic analyses based on the core genomes of *P. aeruginosa* isolates has shown that the vast majority of isolates can be sub-divided into two major phylogenetic subgroups: group 1, which includes the commonly studied strains PAO1, LESB58 and DK2; and group 2, which includes strain PA14 [23].

P. aeruginosa virulence factors

Despite its status as an opportunistic pathogen that rarely causes infections in healthy individuals, *P. aeruginosa* genomes carry a multitude of virulence factors (Table 1) controlled by complex regulatory circuits [24] that are capable of acting in a combinatorial fashion to colonize and injure host tissue [25].

The vast and complicated global regulatory systems employed by *P. aeruginosa* play an important role in regulating virulence during infection. Quorum-sensing (QS) systems have been shown to play a role in regulating the production of numerous virulence factors, including pyocyanin, hydrogen cyanide, elastases A and B, and alkaline protease [24]. *P. aeruginosa* utilizes four distinct QS systems in order to regulate gene expression [26]. Two acylhomoserine lactone (AHL) systems, *las* and *rhl*, rely on feedback loops mediated by *N*-(3-oxododecanoyl)-homoserine lactone (3O-C12-HSL) and *N*-butyrylhomoserine lactone (C4-HSL), respectively. The quinolone-mediated PQS system relies on the compound 2-heptyl-3-hydroxy-4-quinolone. The IQS system is capable of integrating stress cues from the environment with the QS network via secretion and uptake of the molecule 2-(2-hyd roxyphenyl)-thiazole-4-carbaldehyde [27]. Genes regulated by AHL QS systems respond with varying specificity to 3O-C12-HSL and C4-HSL, with some responding equally well to both signals. The genes regulated by *las* and *rhl* make up a large portion of the average *P. aeruginosa* genome (~10%) [28]. There is evidence that both the PQS and IQS systems interact with the AHL QS systems to form a complex and multi-layered signalling system that regulates virulence factors in *P. aeruginosa* [27].

Generally, *P. aeruginosa* isolates can be sorted into two groups (pathotypes) based on mode of toxicity via secreted toxins of the type III secretion system (T3SS); those which secrete exotoxin S (ExoS) and act to cause invasive infections, and those which secrete exotoxin U (ExoU) and cause acute cytotoxicity in host cells [29]. For the most part, those genomes encoding *exoS* fall into phylogenetic group 1 and those encoding *exoU* are in phylogenetic group 2. Most isolates have a copy of only one of these two genes although presence of the gene does not guarantee secretion *in vitro* [30]. Strains of *P. aeruginosa* carrying both or neither of the *exoS* and *exoU* genes are far less common than those containing one or the other and are often considered atypical [31].

ExoS acts with dual functional enzymatic activity to affect two host Ras-related GTP binding proteins. Host cell Ras is inactivated through ADP ribosylation, whilst GTPase activating protein (GAP) activity at the N-terminus of ExoS inactivates Rho proteins [32]. Meanwhile, ExoU has acute

Table 1. Important *P. aeruginosa* virulence factors and their role in pathogenicity

Virulence factor	Secretion system	Host target	Role in pathogenicity
Alginate			Extracellular polysaccharide. Overproduced in mucoid strains often isolated from chronic pulmonary infections. Participates in the production of biofilms. Treatment with imipenem has been shown to induce expression of alginate and leads to thickening of biofilms [89]. Other polysaccharides (Pel and Psl) can also contribute to biofilm formation [90].
Alkaline protease	Type I	Complement proteins	50.4 kDa zinc-dependent metalloprotease. Type I secretion of alkaline protease requires the products of *aprD*, *aprE* and *aprF*. *aprI* is believed to cause intracellular alkaline protease inhibition in order to avoid intracellular toxicity [91].
Elastase A (LasA)	Type II	Matrix proteins	20 kDa metallopeptidase, also known as staphylolysin is secreted as a proenzyme before becoming activated extracellularly. LasA acts with restricted specificity, predominantly at glycine-glycine peptide bonds, but also increases the elastinolytic activity of LasB [92].
Elastase B (LasB)	Type II	Matrix proteins	33 kDa zinc-metalloprotease derived from *lasB*-encoded 52 kDa precursor protein, which undergoes autocatalytic cleavage. The 18 kDa propeptide remains associated with the mature protease to prevent premature proteolytic activity of the enzyme whilst in the cell's periplasm [91].
ExoA	Type II	Cell structure	66 kDa protein member of the AB toxin family formed of two domains. Domain A providies enzymatic activity and domain B acts as a cell binding subunit. In host cell cytosol, elongation factor 2 undergoes catalytic ADP-ribosylation by ExoA which inhibits protein synthesis and induces apoptosis [93].
ExoS	Type III	Cell structure	49 kDa bi-functional cytotoxin. The N-terminal encodes a RhoGAP domain and the C-terminal an ADP-ribosylation domain [94].
ExoT	Type III	Cell structure	Bifunctional toxin. GTP-ase activating protein activity and ADP ribosyl transferase activity. Ribosylates CT10 regulator of kinase (CRK) I and CRKII adaptor proteins [95].
ExoU	Type III	Cell structure	74 kDa hydrophilic protein with broad-range lipase activity. Injected into the host cell via T3SS leads to rapid damage to the cell membrane and organelles with cell lysis [33].
ExoY	Type III	Cell structure	Secreted adenyl cyclase. Increases concentration of intracellular cAMP in host cells, with disruption of actin cytoskeleton and increased endothelial permeability [95].
Flagella		Cell surface	Major surface adhesion. Elicits strong NFκB-mediated inflammatory response via signalling through toll-like receptor (TLR) 5 and a caspase-1-mediated response through Nod-like receptor, Ipaf. Provides bacterium with swimming motility in liquid [96].
Hydrogen cyanide		Mitochondria	Effects of hydrogen cyanide (HCN) on host cells varies depending on cell type and oxidative stress; leading to either apoptosis or necrosis. HCN acts to increase generation of cellular reactive oxygen species in host cells. HCN is produced from glycine in a reaction catalysed by HCN synthase under low oxygen conditions [97].
Nucleoside diphosphate kinase (NDK)	Type I	Macrophages	Catalyses the exchange of phosphate groups between nucleoside diphosphates. NDK induces expression of IL-1α and IL-1β in combination with signals initiated by bacterial flagellin [98].
Phospholipase C	Type II	Cell membranes	Haemolytic phospholipase C cause vascular permeability, end organ damage, and death when administered to mice in high doses. Production is induced by phosphate starvation and may act in phosphate-scavenging pathways in Gram-negative infections in humans in which circulating phosphate levels are sub-optimal for bacterial growth [99].
Protease IV	Type II	Complement proteins	Lysine-specific endoprotease. Protease IV is capable of inactivating a range of host defences including fibrinogen, plasminogen, immunoglobulin G, and complement proteins○ C1q and C3[100]. Initially protease IV is expressed in the cytoplasm as a 48 kDa pre-proenzyme, then cleaved to a 45 kDa proenzyme, and finally cleaved to a 26 kDa mature protease upon secretion. Transcription of the *piv* gene which encodes protease IV is strongly induced by quorum sensing [101].
P. aeruginosa small protease (PASP)	Type II	Structural proteins	18.5 kDa protein capable of cleaving type I and IV collagens. Exists in an inactive form in the cytoplasm and periplasm but becomes active after secretion. PASP has been shown to induce PMN migration in the corneal stroma [42].
Pyocyanin		Widespread toxicity	Redox-active phenazine inhibits host cell respiration, ciliary function, and epidermal growth; disrupts calcium homeostasis and induces apoptosis in neutrophils. Production is partly controlled by oxidative stress response regulator, OxyR and is thought to play a protective role against phagocytic cells [102].

Continued

Table 1. Continued

Virulence factor	Secretion system	Host target	Role in pathogenicity
Pyoverdine		Cellular iron	Siderphore. Little free iron is available in the host environment so pyoverdine acts to sequester iron from host depots [103]. Iron-bound pyoverdine acts as a signalling molecule and interacts with *Pseudomonas* cell receptor FpvA, causing upregulation of exotoxin A, endoprotease and pyoverdine itself [104].
Rhamnolipid			Surfactant. Participates in the maintenance of uninhabited channels surrounding biofilm communities which serve to provide nutrients and oxygen to the colonies of bacteria [105]. Biofilms can form on implants and on dead or living tissue and are inherently difficult to eradicate with antibiotics due to the inability of antibiotic molecules to penetrate the extracellular matrix.
T3SS translocation apparatus		Cell surface	*P. aeruginosa* utilizes three proteins for translocation; PopB, PopD and PcrV. All three proteins are required for pore formation but PopB also plays a role in *P. aeruginosa* pathogenicity independently from effector proteins. PcrV is located at the end of the T3SS needle complex and acts as a platform for PopB and PopD to form the translocation pore [95].
Type IV pili		Cell surface	Major surface adhesion molecules providing twitching motility over moist surfaces. Signal transduction mechanism requires attachment of type IV pili to solid surface, retraction of pilus, and signal transduction through the Chp chemo-sensory system (activates cAMP production and transcription of hundreds of genes, including key virulence factors) [106].

cytolytic activity, which involves targeting of the host cell membrane via phospholipase [33]. Lysis mediated by ExoU is rapid and has been shown to affect mammalian macrophages, epithelial cells and fibroblasts *in vitro* [34–37]. In a previous study utilizing a small panel of isolates it was concluded that there was no significant association between carriage of either *exoS* or *exoU* and isolates collected from keratitis cases [38]. However, in later studies it has been shown that a higher proportion of keratitis infections are caused by isolates carrying the *exoU* gene encoding cytotoxic ExoU, when compared to other kinds of infections [39–41]. In the large-scale Steroids for Corneal Ulcers Trial (SCUT) there was shown to be a significant difference in mean infiltrate/scar sizes between patients infected with invasive (4.66 mm) and cytotoxic (3.61 mm) *P. aeruginosa*. Further analysis of patients in the trial also showed differences in clinical outcomes 3 months after treatment; patients infected with genotypically invasive strains (*exoS*-positive) were shown to present with better visual acuity than those patients infected with cytotoxic strains (*exoU*-positive) but showed less improvement in visual acuity at 3 months. The trial's main purpose was to investigate the potential clinical use of adjunctive topical corticosteroids for the treatment of bacterial keratitis. Further investigation revealed that the corticosteroid treatment had differential effects on ulcers caused by cytotoxic and invasive *P. aeruginosa* [31].

Alongside exotoxins, *P. aeruginosa* produces a range of proteases (Table 1), which cause damage to host cells and aid in immune evasion. Protease IV and *P. aeruginosa* small protease (PASP) have been shown to be present and well conserved in the genomes of several isolates from varying clinical sources, with PASP also being identified as a keratitis-associated virulence factor [42–44].

A study of *P. aeruginosa* strains isolated from cases of keratitis infections in the UK, identified the dominant serotypes as O1 and O11 in addition to common genetic features that may confer an advantage to *P. aeruginosa* during an ocular infection [39]. There has been shown to be a significant correlation between serotype O11 and carriage of the *exoU* gene (Table 1) [30, 39, 45]. Twitching motility, attributed to the type IV pili (Table 1), is implicated in corneal virulence; mutants of *P. aeruginosa* that do not exhibit twitching motility have been shown to have a reduced ability to colonize the cornea of mice vs. strains with unaffected twitching motility [46]. In a further study of UK keratitis *P. aeruginosa*, a large number of isolates were shown to carry an uncommon group II *pilA* gene, suggesting that carriage of particular alleles of *pilA* may be advantageous during keratitis infections [40]. Some of these and other virulence factors have been associated with poorer clinical outcomes in patients [47].

P. aeruginosa keratitis – host response

Corneal infection caused by *P. aeruginosa* has been shown to be associated with significantly worse visual acuities than infection with other bacterial pathogens [48]. A major hallmark of *P. aeruginosa* keratitis is presentation of a ring abscess (Fig. 1), in which a ring-shaped accumulation of polymorphonuclear neutrophils (PMN) surround the central lesion; serrated and satellite lesions are also associated with *P. aeruginosa* keratitis, although the former can appear at first glance to be as a result of a fungal infection [47]. Much of the damage caused to the eye during microbial keratitis is due to the overstimulation of the host immune system by foreign cells in the eye. The cornea exhibits a certain amount of immune privilege; there must still be a system in place by which the eye can detect and defend against potentially harmful microbes. In the cornea, macrophages and dendritic cells play an important role in initiating the innate immune response. The expression of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors

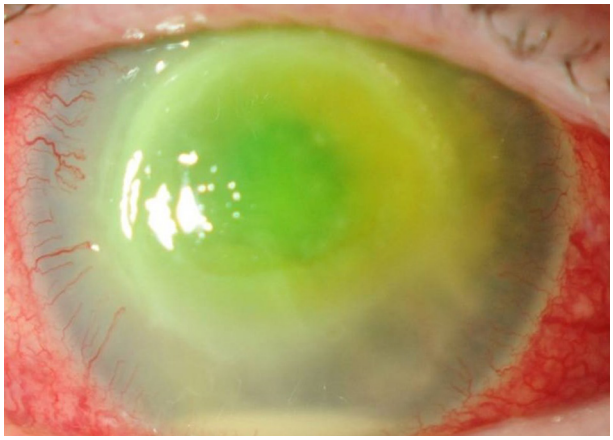

Fig. 1. *P. aeruginosa* keratitis. Note ring shaped abscess and hypopyon (presence of inflammatory cells in the anterior chamber of the eye).

(nucleotide-binding oligomerization domains; NLRs), allows host recognition of conserved pathogen structures known as pathogen-associated molecular patterns (PAMPs). Activation of PRRs results in production of a cascade of inflammatory cytokines such as IL-1, IL-6 and IL-8 via NF-κB [49]. TLR4 and TLR5 on macrophages recognize the flagellin and lipopolysaccharide (LPS) of *P. aeruginosa* and initiate a myeloid differentiation primary response gene 88 (MyD88) mediated pro-inflammatory pathway [50]. Pre-treatment with flagellin has been shown to act protectively in mouse models of keratitis infection by dampening the upregulation of IL-24, which suppresses early mucosal immune responses and leads to increased disease severity [51]. Stimulation of the immune inflammatory response leads to corneal damage and subsequent scarring and vision loss. Prolonged recruitment of PMN and other white blood cells [52] is associated with corneal scarring and there is evidence in mice that a reduction in the recruitment of PMN to the cornea leads to a significant reduction in tissue damage [53]. It is unclear, however, whether a reduction in the PMN response is associated with greater tissue invasion and pathogen retention in the cornea.

As well as the initial inflammatory cascade, there are several factors protecting the eye as an integral part of the innate immune system. These include chemical compounds, which are always present in the tear fluid and aid in generalized protection from microbial pathogens. Iron is an essential nutrient for microbes and so lactoferrin present in the tear fluid sequesters iron away from any potential pathogens. Peptidoglycan in the bacterial cell wall can be cleaved by the enzyme lysozyme, and membrane lipids present in certain bacteria can be cleaved by secretory phospholipase A2(70). Other factors are also produced by cells of the ocular surface upon detection of bacteria, including defensins, which create holes in bacterial cell membranes, and arachidonic acid metabolites, which can further help recruitment of white blood cells.

Because there are limits to the understanding of molecular and cellular reactions in the eye that can be achieved by the study of human subjects, *in vitro* cell line and *in vivo* animal models have been developed, albeit with limitations that reduce their relevance to human infections. To study the pathogenesis of contact-lens-associated microbial keratitis, contact-lens models have been developed in mice [54], rats [55, 56], rabbits [57] and guinea pigs [58]. The supply of contact lenses for such models is often limited, reducing the numbers available for experimentation. Models using larger animals, such as rabbits, often involve surgery on the subject to close the eyelid in order to hold the lens in place on the eye. In models using guinea pigs it was demonstrated that inflammatory events occurred in the eye but did not lead to the development of microbial keratitis [54].

Membrane-localized reporters were used in the mouse contact-lens model to investigate the cellular structural changes that occur in the cornea as a result of extended contact-lens use [54]. After 14 days of continuous wear, vesicles were observed in the external layers of the corneal epithelium. Keratocytes in the stroma were shown to have an altered morphology, with jagged edges, compared to smooth appearance of those in the controls. The presence of highly motile cells moving throughout the stroma was also observed as early as 5 days after the implementation of the contact lenses. As well as the cellular morphology changes observed following extended contact lens wear, there was also shown to be increased neutrophil recruitment into the corneal stroma associated with interleukin 1R (IL-1R) and MyD88 [54]. Long-term, continuous contact-lens use is a known risk factor for complications in humans, so it is important to ascertain if behaviours such as removing contact lenses for a short period of time would be likely to reduce neutrophil recruitment and thus reduce the potential for damage to be caused to the eye by a prolonged inflammatory response.

ANTIBIOTIC RESISTANCE

Advice for the treatment of bacterial keratitis from The Royal College of Ophthalmologists states that initial treatment should be with a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria [59]. However, in recent years it has been observed that to improve clinical outcomes a more targeted treatment is often required [60]. It has been shown that there is a relationship between the MIC of the antimicrobial and the healing time of the corneal ulcer. The lower the MIC, the better the clinical outcome [60]. Rapid identification of the likely causative bacteria combined with clinical acumen enables selection of the appropriate antimicrobial. Isolation of the bacteria allows the MIC to be determined for selected and available antibiotics. In the UK, first-line treatment is generally with a topical fluoroquinolone such as ciprofloxacin, ofloxacin, levofloxacin or moxifloxacin. Prolonged use of aminoglycosides such as gentamicin results in host cell toxicity and potential damage to the corneal epithelium, which may prolong healing time [59]. In cases where first-line treatment is not optimum or fails, or if drug resistance is identified, either switching to an alternative antibiotic or combination therapy may be

required. In cases where combination therapy is required it is important to ensure that additional antibiotic combinations provide additive or synergistic effects.

P. aeruginosa is considered a major global health concern due to increasing levels of resistance to disinfectants and antibiotics. Multidrug-resistant (MDR) strains of *P. aeruginosa* are responsible for approximately 13% of *P. aeruginosa* infections (*n*=6700) and cause more than 400 deaths in the USA annually [61]. *P. aeruginosa* regularly forms biofilms during infection, which confers drastically increased resistance to antibiotics, even if the strains are susceptible when grown planktonically [62]. Antibiotics that are clinically useful, such as carbapenems, fluoroquinolones and aminoglycosides, have been shown to effectively kill *P. aeruginosa* in planktonic growth but can induce more severe biofilm production when the bacteria are exposed at sub-inhibitory concentrations [63]. As bacteria living in a biofilm community are often exposed to sub-inhibitory concentrations of antibiotics when treatments are administered, this may contribute to the exacerbation of chronic infections by increasing the robustness of existing biofilms or encouraging the spread of the infection. Much of the intrinsic resistance conferred to *P. aeruginosa* is due to low membrane permeability and a range of multidrug efflux pumps, particularly the MexAB-OprM and MexXY-OprM systems [64, 65].

In work carried out to investigate antibiotic susceptibility and effects of treatment in keratitis isolates, antibiotic resistance has been shown to be relatively uncommon. In 158 *Pseudomonas* spp. isolates collected from clinics in the UK, mean MIC_{90} for commonly used topical antibiotics were all equal to or lower than the systemic breakpoints (Table 2) [60]. A large-scale review was also carried out to determine the reported rates of resistance to those antibiotics most commonly used in the treatment of microbial keratitis (ciprofloxacin, β-lactam group cephalosporins and gentamicin) among bacterial isolates from microbial keratitis cases in Australia. It showed that rates of resistance in ocular isolates of *P. aeruginosa* and *S. aureus* were low [66]. Higher rates of resistance have been reported in the USA and India [66]. It is possible that increased resistance occurs in these countries as they impose fewer regulations on the use of antibiotics in livestock, which may contribute to increasing incidence of antibiotic resistance in bacteria in environmental reservoirs, from which it is believed that many of the bacteria that infect eyes are derived. In India, there are also concerns about the availability of antibiotics without prescription ('over the counter') and their inappropriate prophylactic use, which may also contribute to increased antibiotic resistance [66]. In general, resistance to the most commonly used drugs in the treatment of microbial keratitis is uncommon in both *P. aeruginosa* and *S. aureus*.

Table 2. Mean MIC_{90} values for *Pseudomonas* spp. isolated in the UK from Kaye *et al.* [60] alongside systemic breakpoints taken from EUCAST guidelines [107]. Breakpoint data for *Pseudomonas* spp. and ofloxacin were not available in the EUCAST document

Antibiotic	Breakpoint (mg l^{-1})	MIC_{90} (mg l^{-1})
Ceftazidime	8	2
Gentamicin	4	2
Amikacin	8	4
Ciprofloxacin	0.5	0.5
Ofloxacin	Not available	1.5

Antibiotic resistance in *P. aeruginosa* is often conferred by mutations leading to, for example, up-regulation of resistance-related genes (efflux pumps, β-lactamases) or reduced entry (porins, such as OprD). However, mobile genetic elements (MGEs) are often associated with MDR strains of *P. aeruginosa*, especially in hospital settings [67]. The three most commonly used antibiotic classes in the treatment of ocular infections are beta-lactams, aminoglycosides and fluroquinolones, and genes conferring resistance to all antibiotics from all three of these classes have been shown to be transmissible by MGE between bacterial strains [67]. *P. aeruginosa* isolates from keratitis infections in India were shown to have additional resistance genes, including *qacEΔ1*, a quaternary ammonium compound (QAC) resistance gene [68]. Earlier research showed that MDR strains of *P. aeruginosa* sometimes carry *qac* resistance genes but that these genes do not always confer increased resistance to QACs, even in isolates with increased antibiotic resistance [69].

Treatment of microbial eye infections most commonly utilizes a combinatorial approach, with clinicians most commonly prescribing dual therapy targeting both Gram-negative and Gram-positive pathogens. This treatment usually consists of a β-lactam and an aminoglycoside as a first course of action upon the patient presenting to their health practitioner [66]. As such, the effects of combinatorial treatments against keratitis *P. aeruginosa* isolates have also been investigated. Measuring the fractional inhibitory concentration (FIC) allowed classification of combinations of antibiotics as synergistic, additive or antagonistic. No consistent antagonistic effects were found, and the only synergistic effect was detected during combinatorial treatment with meropenem and ciprofloxacin. This is in comparison to treatment of *S. aureus* isolates, for which synergistic or additive effects were demonstrated for four combinations of antibiotics in 60–80% of isolates [70].

Resistance to disinfectants and preservatives

Patient hygiene and compliance also plays a major role in the development of contact-lens-associated keratitis infections, and the importance of effective cleaning solutions for non-daily contact lenses cannot be understated. There are a wide range of commercially available contact lens disinfecting solutions, both branded and generic, that utilize different active ingredients (Table 3) in order to prevent microbial contamination of contact lenses.

QACs are among the most commonly used active ingredients in contact-lens disinfecting solutions, primarily chosen for their effectiveness and ease of use, but are also used in a range of household products including shampoo, sun creams and lotions, make-up remover and hand sanitizer [71].

Table 3. Commonly used, commercially available contact-lens disinfecting solutions and their active antimicrobial ingredients. Ingredients and information sourced from work by Lin *et al.* [76] and Johnston *et al.* [108]. All ingredient concentrations listed refer to % (v/v)

Disinfecting solution	Active ingredients	
Boston Simplus (Bausch and Lomb)	Chlorhexidine gluconate (0.003%), polyaminopropyl biguanide (0.0005%)	* +
Boston Advance (Bausch and Lomb)	Chlorhexidine gluconate (0.003%) Polyaminopropyl biguanide (0.0005%) Ethylenediaminetetraacetic acid (0.05%)	*
Opti-Free GP (Alcon)	Polyquarternium-1 (0.0011%) Ethylenediaminetetraacetic acid (0.01%)	*
Opti-Free RepleniSH (Alcon)	Propylene glycol Polyquarternium-1 (0.001%) Aldox (0.0005%)	+
Menicare GP (Menicon)	Benzyl alcohol (0.3%) Ethylenediaminetetraacetic acid (0.5%)	*
Lobob (Lobob Labs)	Benzyl alcohol (0.25%) Benzalkonium chloride (0.01%)	*
AQuify (Ciba Vision)	Polyhexanide (0.0001%)	+

*solutions used by Lin *et al.* [76].
+solutions used by Johnston *et al.* [108].

Traditionally, disinfection of contact lenses used hydrogen peroxide-based solutions that required two steps to be carried out by the user: disinfection using hydrogen peroxide, followed by neutralization of the active ingredient with a separate solution before applying the contact lens. Multipurpose 'no rinse' solutions (Table 3) have become increasingly popular with consumers due to ease of use. Many contact-lens practitioners and opticians tend to recommend solutions that are easier to use to maximize user compliance and therefore reduce the chance of infections and other contact-lens-associated complications. The method of use for no rinse multipurpose solutions, many of which are also 'no rub' (i.e. the contact lens does not need to be rubbed between the fingers in order to ensure removal of any possible attached microbes), means that micro-organisms, bacteria in particular, may be exposed to sub-inhibitory concentrations of the active ingredient for an extended period of time. This allows for selection of those remaining bacteria with increased MIC values and an increased likelihood of development of resistance to the disinfectant. Similarly it has also been shown that use of QACs can lead to increased fixation of novel genetic elements, which can lead to the acquisition and spread of resistance to antibiotics [72]. The *qac* family of genes (*qacC*, *qacG*, *qacH* and *qacJ*), named for their role in resistance to QACs, are found across several bacterial genera and have been shown to play a role in resistance to a range of cationic compounds, including intercalating dyes, diamidines and biguanides [73–75]. The proteins encoded by the *qac* genes are involved in low-specificity multidrug efflux pumps [75].

The antibacterial activities of five major multipurpose contact-lens disinfecting solutions were tested against *P. aeruginosa* PAO1 and methicillin-resistant *S. aureus* (MRSA) TCH1516 using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method. The solutions that were most active were not the same for *P. aeruginosa* and *S. aureus*; Boston Simplus (Bausch and Lomb, USA) was the most antistaphylococcal of the solutions, whilst Menicare GP (Menicon, Japan) was shown to have the highest antipseudomonal activity [76]. Further to this, the MIC for Boston Simplus against MRSA was 1.5% whereas the MIC for Menicare GP against *P. aeruginosa* was 23%. All of the disinfecting solutions were shown to be more effective against MRSA than *P. aeruginosa*, and the same results were observed when MIC testing was repeated with three *S. aureus* and three *P. aeruginosa* clinical isolates [76]. When tested alone, the most effective preservative against *P. aeruginosa* was shown to be ethylenediaminetetraacetic acid (EDTA), with an MIC of 2500 p.pm (vs. 300 ppm against *S. aureus*), but EDTA was shown to act synergistically against *P. aeruginosa* with chlorhexidine gluconate (CHD). When combined, a solution containing 4 ppm CHD (~1/4 MIC) and 300 ppm EDTA (~1/8 MIC) was sufficient to eradicate *P. aeruginosa*, reducing bacterial numbers by >4 $\log_{10}$ in quantitative 2 h killing assays [76].

It has been suggested that constant exposure to biocides across domestic, healthcare and industrial environments at a range of concentrations, from sub-inhibitory to inhibitory, also contributes to the development of resistance to both antibiotics and disinfectants among bacterial populations [77]. However, there is conflicting evidence, which indicates that this type of co-adaptation to increased survival in the presence of both antibiotics and disinfectants is uncommon and of little clinical significance [78, 79].

FUTURE TREATMENTS AND DEVELOPMENTS

With growing concerns about the rise of antibiotic resistance in bacterial species, particularly in *P. aeruginosa*, the need for new or improved treatments for all manner of bacterial infections is greater than ever. Bacteriophages were first identified in the early twentieth century, but research into the potential therapeutic uses of phage did not start in earnest until the 1920s and was largely abandoned as a treatment option following the discovery of penicillin and the dawn of the antibiotic era. Driven by the emergence of MDR strains of pathogenic bacteria, research into phage therapy has experienced a resurgence in recent years. Phage have been shown to effectively kill bacteria and improve disease outcomes in a range of animal models and infections [80]. Of particular interest is the topical application of phage preparations for the treatment of keratitis. Fukuda *et al.* [81] infected the corneas of mice with a clinical *P. aeruginosa* strain via topical administration following damage to the cornea. Mice subsequently treated with KPP12 phage were shown to undergo a drastically less severe disease course than those mice treated

with the vehicle (mock-treated). Histopathological examination of corneas from treated and mock-treated mice revealed structural differences within the eye following infection and subsequent treatment, with mock-treated mice suffering more severe structural damage. Further examination showed that bacteria were present in almost undetectable loads in treated mouse corneas, whereas *P. aeruginosa* was shown to be present in high numbers in the abscesses of mock-treated mice. The improvements in disease severity elicited by this phage treatment against severe *P. aeruginosa* keratitis infection show promise for the future of topical phage therapy as an alternative to current antibiotics.

There has been increasing interest in the development of substances with antipathogenic (or anti-virulence) properties in both healthcare-associated and industrial settings which, by definition, do not kill bacterial pathogens but instead act to reduce damage to both products and human health [82–86]. In combination with antibiotics, these substances may offer an alternative way to reduce the morbidity associated with *P. aeruginosa* keratitis. By investigating antipathogenic treatments, focus can be shifted away from the struggle to develop new and effective ways to kill bacteria and can instead focus on reducing harm by any means possible. Some such treatments under development target the inflammasomes of the immune system, which play an important role in inflammation of the eye during infection, leading to destruction of tissue [87]. In particular, nod-like receptors (NLR) P1, NLRP3, NLRC4 and the AIM2 (absent in melanoma 2) protein have been shown to play a role in increased severity in eye diseases. NLRP3 generally plays a protective role in fighting infection in other parts of the body, particularly during lung infection, but in the eye it acts to recruit cytokines and chemokines, which increase inflammation [87]. Drugs that inhibit caspase-1 have been used as an effective adjuvant therapy in a mouse model of keratitis infection to reduce disease severity. Caspase-1 acts to cleave prointerleukin-1β (IL-1β) and prointerleukin-18 (IL-18) to their active forms, which go on to contribute to inflammation. A caspase-1 inhibitor can be effectively used as an adjuvant therapy alongside ciprofloxacin to reduce the severity of corneal inflammation during infection, as well as a significant reduction in recruitment of PMNs. Mice in this study were also infected with a ciprofloxacin-resistant strain of *P. aeruginosa* and improved clinical scores were observed following treatment with the caspase-1 inhibitor and ciprofloxacin combined treatment [88].

The antipathogenic effects of essential oils are also of interest, commonly derived from foodstuffs, and their effects on QS systems and biofilm formation. The non-volatile essential oil 6-Gingerol, derived from ginger root, has been reported to have anti-QS and anti-biofilm effects comparable to the synthetic compound furanone C-30 against *P. aeruginosa*, as well as anticancer, anti-inflammatory and analgesic effects in humans [82]. Flavonoids are naturally produced plant metabolites that have been shown to reduce *P. aeruginosa* biofilm formation by inhibition of the LasR QS system [85]. The molecules act by binding to the LasR ligand binding domain and preventing binding of the protein to DNA. *In vivo* the flavonoids were shown to inhibit transcription of *rhlA* and reduce pyocyanin production [86]. Whilst antipathogenic substances are unlikely to offer a single solution to the problem of antibiotic resistance, there is potential for development of antipathogenic substances as adjuvants to antibiotic treatment, both to reduce morbidity to patients and reduce the burden of antibiotic resistance on healthcare systems worldwide.

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Conflicts of interest
The authors declare that there is no conflicts of interest.

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