**DECLARATION OF COMPETING INTEREST**

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**ABSTRACT**

Bacterial keratitis is a common corneal infection that is treated with topical antimicrobials. By the time of presentation there may already be severe visual loss from corneal ulceration and opacity, which may persist despite treatment. There are significant differences in the associated risk factors and the bacterial isolates between high income and low- or middle-income countries, so that general management guidelines may not be appropriate. Although the diagnosis of bacterial keratitis may seem intuitive there are multiple uncertainties about the criteria that are used, which impacts the interpretation of investigations and recruitment to clinical studies. Importantly, the concept that bacterial keratitis can only be confirmed by culture ignores the approximately 50% of cases clinically consistent with bacterial keratitis in which investigations are negative. The aetiology of these culture-negative cases is unknown. Currently, the estimation of bacterial susceptibility to antimicrobials is based on data from systemic administration and achievable serum or tissue concentrations, rather than relevant corneal concentrations and biological activity in the cornea. The provision to the clinician of minimum inhibitory concentrations of the antimicrobials for the isolated bacteria would be an important step forward. An increase in the prevalence of antimicrobial resistance is a concern, but the effect this has on disease outcomes is yet unclear. Virulence factors are not routinely assessed although they may affect the pathogenicity of bacteria within species and affect outcomes. New technologies have been developed to detect and kill bacteria, and their application to bacterial keratitis is discussed. In this review we present the multiple areas of clinical uncertainty that hamper research and the clinical management of bacterial keratitis, and we address some of the assumptions and dogma that have become established in the literature.

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1. **INTRODUCTION**
	1. ***Definition of the disease***
	Several terms have been introduced to describe corneal disease due to a suspected pathogen. Ulcerative keratitis and suppurative keratitis are imprecise descriptive terms. Infectious keratitis is also used although the infection is not readily transmissible to other individuals. We prefer the term microbial keratitis, which includes any infection of the cornea by a suspected microorganism or infective agent (bacterium, fungus, protozoa, virus, prion) present either in isolation or in combination. The primary focus of this review is suspected bacterial keratitis. Surprisingly, there are no agreed diagnostic criteria for this disease because, in contrast to many other diseases, there is not a reliable gold reference standard, and therefore the accuracy of tests for suspected bacterial keratitis cannot be verified [(Korevaar et al., 2019)](https://paperpile.com/c/Vdx3Lu/vivY5). It is not always possible to discriminate between the true bacterial pathogen and a contaminant, or a negative culture from the absence of infection. This likely leads to an underestimate of the disease prevalence and an overestimate of the value of investigation [(Umemneku Chikere et al., 2019)](https://paperpile.com/c/Vdx3Lu/ppry). These uncertainties have reduced the generalisability of published clinical studies. Of particular relevance to clinical care, the aetiology of disease in the 20%-70% of cases of corneal disease compatible with bacterial keratitis in which investigations are negative is unknown [(Ung and Chodosh, 2021)](https://paperpile.com/c/Vdx3Lu/kSBs), with an absence of objective data to guide management.
	2. ***Clinical description of bacterial keratitis***
	Bacterial keratitis is typically characterised by a painful epithelial defect with associated signs of corneal stromal inflammation and ulceration. The infected eye is usually red from a diffuse conjunctivitis, with episcleritis and rarely scleritis in severe cases. There is often a localised corneal opacity with thinning (corneal melt), accompanied by an anterior uveitis, fibrinous exudate or a hypopyon. Corneal melt may progress to a descemetocele and perforation, but endophthalmitis is uncommon. Occasionally patients can lose an eye from microbial keratitis, particularly in older patients [(Butler et al., 2005)](https://paperpile.com/c/Vdx3Lu/x6Ef). Residual signs may include a corneal scar with vascularisation and thinning, or an adherent leukoma, with visual loss from opacity and irregular astigmatism.
	3. ***Epidemiology of bacterial keratitis***
		1. ***Global burden of blindness from bacterial keratitis***
		There is currently no accurate estimate of the global burden of blindness from bacterial keratitis. The prevalence cannot be reliably inferred from data for non-trachomatous corneal opacification, which is the sixth leading cause of global blindness, accounting for 3.2% (80% UI 0.5-7.2) of the 36x106 cases, because these data almost exclusively report cases grouped as microbial keratitis [(Flaxman et al., 2017; GBD 2019 Blindness and Vision Impairment Collaborators and Vision Loss Expert Group of the Global Burden of Disease Study, 2021; Resnikoff et al., 2004)](https://paperpile.com/c/Vdx3Lu/dDYbi%2BrsUDH%2BSEee). Data from low and middle income countries, which accounts for three quarters of the global population, is particularly sparse. Published figures for the incidence of MK include 113 per 100,000 population-year in South India [(Gonzales et al., 1996; Srinivasan et al., 1997; Whitcher and Gonzales, 1998)](https://paperpile.com/c/Vdx3Lu/hY7e%2BUPYW%2BBFLw) and 799 per 100,000 population-year from Nepal [(Upadhyay et al., 2001)](https://paperpile.com/c/Vdx3Lu/5VGY). The proportion of pathogens (virus, bacteria, parasite, fungus) also varies widely between geographic regions, with fungal keratitis particularly prevalent in low and middle income countries and equatorial regions [(Brown et al., 2021)](https://paperpile.com/c/Vdx3Lu/IKrb). A population based study in China estimated the prevalence of past or active infectious keratitis to be 192 (95% CI 171–213) per 100,000 population, with a prevalence of presumed viral keratitis of 110, bacterial keratitis 75, and fungal keratitis 7 per 100,000 population [(Song et al., 2014)](https://paperpile.com/c/Vdx3Lu/6KnV). The incidence figures for MK reported from high income countries since 1995 are much lower with estimates of 4.5-37.7 cases per 100,000 population-year in the US, UK, Australia and Taiwan [(Stapleton, 2021)](https://paperpile.com/c/Vdx3Lu/c4co), which follows a marked increase in cases from the 1970’s associated with the widespread introduction of contact lens wear [(Erie et al., 1993)](https://paperpile.com/c/Vdx3Lu/TGNR). Importantly, these estimates are usually based on the results of laboratory records of positive corneal cultures, which may significantly underestimate the true incidence (see section 2.1).

		The frequency of isolation of probable bacterial pathogens is usually derived from retrospective epidemiological data, with implications for the development of regional treatment protocols, the interpretation of clinical trials [(Srinivasan et al., 2012)](https://paperpile.com/c/Vdx3Lu/wbEK), and the identification of emerging risk factors [(Erie et al., 1993)](https://paperpile.com/c/Vdx3Lu/TGNR). The most common isolates are coagulase negative Staphylococci (CoNS) (24%–46%), *Staphylococcus aureus (S. aureus)* (5–36%), *Streptococcus spp.* (7%–16%), *Pseudomonas aeruginosa (P. aeruginosa)* (5%–24%), *Enterobacteriaceae spp*. (15%), *Corynebacterium spp*. (14%), and *Propionibacterium spp.* [(Stapleton, 2021; Teweldemedhin et al., 2017; Ung et al., 2019)](https://paperpile.com/c/Vdx3Lu/v7Iw%2BDpZD%2Bc4co). *Streptococcus pneumoniae (S. pneumoniae)* and *Nocardia spp.* are also frequently reported in South India, where agricultural trauma is a major risk factor [(Bharathi et al., 2002; Lalitha et al., 2017; Srinivasan et al., 2012)](https://paperpile.com/c/Vdx3Lu/Fx1f%2BwbEK%2BiF6q). Differences in environmental temperature, humidity, occupation, associated viral disease and malnutrition will all affect the local prevalence pattern [(Shah et al., 2011)](https://paperpile.com/c/Vdx3Lu/pJTL). Ideally, all ulcers should be sampled, as the introduction of a threshold ulcer size to justify corneal culture will introduce case selection bias and affect the spectrum of isolates [(A. Lin et al., 2019; Ung et al., 2020)](https://paperpile.com/c/Vdx3Lu/LhFq%2BTDZS). Any effect on prevalence from the use of phenotypic identification as opposed to matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF) are likely to be slight as *Pseudomonas spp*. and *S. aureus* are readily identified with both methods. The epidemiology of antimicrobial resistance patterns to ophthalmic appropriate antimicrobials is less frequently reported in epidemiological studies, although it is of greater utility (section 5.2).
		2. ***Risk factors for infection***
		Bacteria are part of the normal flora of the ocular surface, although it is not known if there is a characteristic microbiome for the cornea. With some possible exceptions, e.g. *Neisseria spp.* pilus-mediated cell attachment [(Tjia et al., 1988)](https://paperpile.com/c/Vdx3Lu/28Eh), and possibly also*Corynebacterium diphtheriae*, *Haemophilus influenzae*, and *Listeria sp*. [(P. Singh et al., 2021)](https://paperpile.com/c/Vdx3Lu/j51V), bacteria that commonly cause keratitis (*P. aeruginosa, S. aureus*) do not normally attach to and penetrate a healthy intact corneal epithelium [(Fleiszig et al., 2020)](https://paperpile.com/c/Vdx3Lu/fdBK). Most infections follow an identifiable intrinsic or extrinsic event that damages the epithelial barrier to allow endogenous or exogenous bacterial invasion. Risk factors include contact lens wear, agricultural trauma and chronic ocular surface disease [(Bourcier, 2003; Chidambaram et al., 2018; Dart et al., 1991; Erie et al., 1993; Jeng et al., 2010; Srinivasan et al., 1997; Stapleton, 2021; Szczotka-Flynn et al., 2021; Upadhyay et al., 2001)](https://paperpile.com/c/Vdx3Lu/hY7e%2B5VGY%2BiTL3%2BX1tZC%2B9YvIl%2BTGNR%2BliZ2%2BsMT0%2Bc4co). In high income countries contact lenses are the major risk factor for infection, with the incidence of presumed microbial keratitis in contact lens wearers of between 24.4-130.0 per 100,000 person years, influenced by the type of lens and regime of wear [(Cheng et al., 1999; Dart et al., 1991; Stapleton, 2021; Stapleton et al., 2008)](https://paperpile.com/c/Vdx3Lu/PxAu%2BeGw9%2B9YvIl%2Bc4co). Contact lens-associated microbial keratitis is strongly associated with Gram negative bacterial infection, especially *P. aeruginosa* [(Alfonso et al., 1986; Dart, 1988; Golden et al., 1971; Liesegang and Forster, 1980; Stapleton et al., 1995; Stapleton and Carnt, 2012)](https://paperpile.com/c/Vdx3Lu/DRuK%2B0HzK%2B7jjK%2ByMjZ%2BKlIG%2BbJgI), which are ubiquitous in the environment [(Stapleton, 2020)](https://paperpile.com/c/Vdx3Lu/jueq). Contamination of the contact lens care system may enable a protective biofilm to form that prevents effective disinfection [(Fleiszig et al., 2020; McLaughlin-Borlace et al., 1998; Wu et al., 2010)](https://paperpile.com/c/Vdx3Lu/lE4F%2BA595%2BfdBK), and inadequate exposure to the contact lens solution may also lead to the selection of *P. aeruginosa* that are resistant to that solution [(Fleiszig et al., 2020; Lakkis and Fleiszig, 2001)](https://paperpile.com/c/Vdx3Lu/fdBK%2B1y3b), increasing the risk of transfer of bacteria to the eye.

		Ocular surface disease is also a major risk factor for the development of bacterial keratitis and for recurrent disease. The majority (around 60–80%) of isolates from these cases are endogenous Gram-positive bacteria such as CoNS, *S. aureus*, *Streptococcus spp.*, and *Corynebacterium spp.* [(Khoo et al., 2020, 2019; Sagerfors et al., 2020)](https://paperpile.com/c/Vdx3Lu/nQbR%2BT5x8%2BPSTj). This may be the result of colonisation by *S. aureus* from contiguous sites e.g. the lid margins, periocular skin, and the anterior nares [(Somerville et al., 2020b)](https://paperpile.com/c/Vdx3Lu/G6fl), *S. pneumoniae* colonisation from nasolacrimal duct obstruction (Chidambaram et al., 2018), or *S. aureus* colonisation in the giant fornix syndrome [(Rose, 2004)](https://paperpile.com/c/Vdx3Lu/mU2Z). Colonisation and recurrent infection are also more prevalent in conditions associated with severe ocular surface disease such as rheumatoid arthritis, Sjogren syndrome, ectodermal dysplasia, Steven Johnson syndrome, graft versus host disease, and atopic keratoconjunctivitis. However, there has been no molecular confirmation that bacterial strains that colonise these contiguous sites are the same strains as those that are isolated from a corneal ulcer. ​​

Immunodeficiency may also increase the susceptibility to infection with an approximately ninefold increased incidence of ulcerative keratitis (mainly viral) in human immunodeficiency virus-positive individuals compared to healthy individuals (238.1 vs. 27.1 per 100,000 person-year) [(Jeng et al., 2010)](https://paperpile.com/c/Vdx3Lu/liZ2). The risk of microbial keratitis is also increased by diabetes, possibly as a result of neurotrophic corneal change or changes in the microbiota [(Chidambaram et al., 2018; Dan et al., 2018; Li et al., 2019; Pan et al., 2016; Wang et al., 2018)](https://paperpile.com/c/Vdx3Lu/iTL3%2BSrMz%2BeHAy%2BaLev%2B5Vk1).

* + 1. ***Temporal changes of keratitis isolates***The spectrum of corneal isolates can change as a result of urbanisation and the introduction of new risk factors. The most important example being the introduction of contact lens wear which paralleled a major increase in the proportion of Gram-negative isolates from cases of keratitis, particularly *P. aeruginosa* [(Alfonso et al., 1986)](https://paperpile.com/c/Vdx3Lu/7jjK). This has had implications for empiric treatments and management. New surgical procedures, such as laser refractive surgery, has been associated with outbreaks of keratitis from pathogens that were previously uncommon such as *Mycobacterium chelonae*, *M. fortuitum*, and *M. abscessus*, that can change the pattern of recorded isolates [(Bostan et al., 2019; Chandra et al., 2001; Freitas et al., 2003; Nascimento et al., 2018; Pacheco and Tam, 2010; Seo et al., 2002; Winthrop et al., 2003)](https://paperpile.com/c/Vdx3Lu/T2IC%2BTkxB%2BJE1s%2BBlvR%2Bc5qP%2BwPqv%2Blfzo). Additional new risk factors for microbial keratitis, particularly in children, include orthokeratology and corneal collagen cross linkage (CXL) [(Maharana et al., 2018; Tzamalis et al., 2019)](https://paperpile.com/c/Vdx3Lu/KcSd%2BzD7S).
		2. ***Outcomes and personal burden of disease***
		The outcome after bacterial keratitis is strongly associated with the isolated microorganism [(Wilhelmus, 1996)](https://paperpile.com/c/Vdx3Lu/ERPt). Virulent organisms such as *Streptococcus pneumonia*, *P. aeruginosa* or *N. meningitidi*s can cause a rapid progression to a total corneal abscess, stromal lysis and corneal perforation. In contrast, with low virulence CoNS species, the infection may be slowly progressive or even self-limiting. It is important to note that outcomes can be determined by virulence factors that can vary significantly within bacterial species. The visual consequence of infection depends on the position on the cornea, with large central ulcers associated with more visual loss, although secondary visual loss from cataract or secondary glaucoma can also occur [(McClintic et al., 2014)](https://paperpile.com/c/Vdx3Lu/Tbwa). The outcome of microbial keratitis is worse in low-income countries compared to high income countries. This is a result of limited access to healthcare, delayed presentation for treatment, a high proportion of fungal infections, concurrent ocular damage from trauma, inadequate first line treatment, and the early empiric use of topical corticosteroid [(Arunga et al., 2019a; Burton et al., 2011; Tuohy et al., 2021; Vajpayee et al., 1999)](https://paperpile.com/c/Vdx3Lu/f50x%2BqiLw%2BP5S7%2BVUUU). Vision loss following microbial keratitis leads to a reduced quality of life [(Arunga et al., 2019b; Y. Li et al., 2014)](https://paperpile.com/c/Vdx3Lu/xy6b%2BpD97) and an associated economic burden from direct and indirect costs [(Collier et al., 2014; Keay et al., 2008; Moussa et al., 2020; Smith and Orsborn, 2012)](https://paperpile.com/c/Vdx3Lu/8tb0%2BuylA%2BrNjv%2Bcd2q).
1. ***WHY IS THE CURRENT SITUATION UNSATISFACTORY***
	1. **Case definition for suspected bacterial keratitis is uncertain**
	Several levels of evidence can help confirm or refute a provisional diagnosis of bacterial keratitis. The first is clinical suspicion, based on the appearance of the lesion and the presence of risk factors for infection. Investigations are then based on samples taken from the corneal lesion for microscopy, culture and antimicrobial susceptibility, polymerase chain reaction (PCR), and, in selected cases, *in vivo* confocal microscopy. However, the utility of investigation for bacterial keratitis is debated because of the perceived low sensitivity of the tests and uncertainty about the extent that investigations influence management decisions.

	For bacterial keratitis a hierarchy can describe test utility: i) the test (e.g. microscopy, culture or PCR) must be able to detect small numbers of bacteria, ii) it should also be accurate with a low level of false positive and false negative results, iii) the result of the test should have the ability to influence patient management, e.g. to start or stop an antimicrobial, iv) this decision should then improve the patient outcome, v) the cost and benefits of the investigation should be justified [(Bossuyt et al., 2012; Fryback and Thornbury, 1991)](https://paperpile.com/c/Vdx3Lu/HPniT%2Bs5JBM). In practice, an investigation that is sensitive and accurate in the laboratory may not be useful in clinical practice. Most reports on test utility only assess diagnostic accuracy (i.e. level ii), but with little discussion of false positive or negative results, or how the result changes clinical management or outcomes. A complicating factor, particularly relevant to bacterial keratitis, is that the results are subject to confirmation bias, with more weight given to a positive result than a negative result. As a result, the overall contribution of investigations to patient outcomes is poorly understood.

	Defining a case of bacterial keratitis for clinical studies is problematic. All investigations are liable to yield a proportion of false positive and false negative results but, without a reliable reference standard, the magnitude of these errors cannot be quantified, and thus the concept of a gold standard definition for bacterial keratitis is untenable. Performing a comparison of test utility by adopting culture as a gold reference standard and calculating sensitivity and specificity with positive and negative predictive values, is also invalid because an accurate estimate of false positives and negatives is integral to these calculations. This uncertainty regarding case definition, and the true status of disease, is especially relevant when interpreting recruitment to clinical trials. As an alternative, a pragmatic criterion would be a consensus opinion of disease status based on a range of indices, including clinical signs, the decision to treat, as well as the results of clinical investigations.
	2. **Poor distinction between pathogen or contaminant**
	There is debate as to when a cultured bacterium constitutes a pathogen, a commensal, or a contaminant, which has clinical consequences. For example, if CoNS (24%–46% of isolates in most studies) are classed as commensals and excluded this will affect both the outcome of clinical trials, the interpretation of the effect of treatment, and the pattern of antimicrobial antimicrobial resistance (AMR). As a corollary the proportion of cases of keratitis of unknown aetiology will increase. As new technologies are introduced, any improved sensitivity of investigation may have the unwanted effect of identifying more isolates of uncertain significance. A reliable method to distinguish a pathogen from a commensal is not currently available, but when more knowledge is gained regarding the corneal microbiome in health and disease, this distinction may become clearer.
	3. **No rapid method to confirm susceptibility of the pathogen at presentation**
	Clinicians base their initial treatment of suspected bacterial keratitis on clinical acumen, exogenous or endogenous risk factors, the availability of effective topical antimicrobials, and relevant data on antimicrobial resistance in the catchment population. This means that the great-majority of treatments are delivered empirically with a subsequent delay of days before the results of investigation might be available to guide further management. In the UK, the recommendations of the O’Neill Report mandated that all antimicrobial prescriptions should be informed by up-to-date surveillance information and a rapid diagnostic test [(O’Neill, 2016)](https://paperpile.com/c/Vdx3Lu/kyjk). New diagnostic techniques and therapies have been developed for the identification of organisms, but there is often no realistic discussion of the route map toward their implementation as a useful tool at the point of first patient contact. These technologies may also be inappropriate for the low- and middle-income countries where there is the greatest burden of disease. Although it is more than 20 years since the first reports of molecular methods for the investigation of suspected microbial keratitis [(Knox et al., 1998)](https://paperpile.com/c/Vdx3Lu/LVVt), there is still no high quality evidence that the introduction of polymerase chain reaction (PCR) or sequencing are more efficient or more reliable than culture for the identification of bacterial pathogens or their resistance to antimicrobials.
	4. **Optimal sampling approach is unknown**​​There is limited evidence as to the best way to sample an ulcer to detect bacteria. Methods vary in their sensitivity, but it is not known if these differences relate solely to the small tissue volumes obtained. Sampling requires tissue and inflammatory material taken from the base or edge of the ulcer without causing additional damage. Direct sampling of infection sequestered beneath an intact epithelium, such as beneath a LASIK flap, may also be necessary. Sampling has been reliant on using an instrument such as a Kimura spatula, blade or the bent tip of a hypodermic needle. This requires patient co-operation and operator skill, with clinician training to ensure correct plating and to reduce the risk of contamination of the sample [(Ngo et al., 2020)](https://paperpile.com/c/Vdx3Lu/Bdkk). Taking a sample with a flexible absorbent device such as a swab or culturette requires less skill and is generally better tolerated. However, they are larger and more difficult to localise to an ulcer and they are not specifically designed to adsorb or release microorganisms. The components of the swab (cotton, calcium alginate, cellulose) may also inhibit the PCR reaction [(Daley et al., 1995; Llabrés and Rose, 1989; Wadowsky et al., 1994; Zasada et al., 2020)](https://paperpile.com/c/Vdx3Lu/bNMy%2BqIjC%2BD2rR%2BbVOc). Nylon flocked swabs have a high yield for collecting bacteria for culture and DNA for molecular studies [(Bruijns et al., 2018; Ozkan et al., 2017; Pakzad-Vaezi et al., 2015)](https://paperpile.com/c/Vdx3Lu/yRL9%2BbDez%2Bq7AH), but there is conflicting evidence as to whether bacterial adsorption and release is better with a saline-moistened swab or a dry swab [(Epley et al., 1998; Rose et al., 2004)](https://paperpile.com/c/Vdx3Lu/ZTSm%2Buoc5). A flexible polytetrafluoroethylene corneal impression membrane placed on the surface of the ulcer is easy to use and a low risk technique that gives equivalent or superior bacterial detection rates compared to scraping [(Bruijns et al., 2018; Kaye et al., 2016; Ozkan et al., 2017)](https://paperpile.com/c/Vdx3Lu/bDez%2Bq7AH%2BxGow). The high detection rates, together with the ease of use, makes it a good option when slit-lamp biomicroscopy or a skilled operator is unavailable [(Brunner et al., 2019)](https://paperpile.com/c/Vdx3Lu/0IAw). It is not known, however, whether the profile of the recovered bacteria differs between sampling techniques or if an inadequate sample is the sole explanation for the high proportion of culture or PCR results that are negative, or whether there are differences between samples taken from the superficial ulcer as opposed to the deeper tissue. Finally, for chronic or unresponsive keratitis, or progressive stromal infiltrate beneath an intact epithelium, a surgical biopsy of tissue adjacent to the ulcer for culture and histology should be considered. It has been reported that in 21%-89% of cases of biopsy may identify an organism that has not been detected on previous culture, and that in 73%-89% of cases the bacterium was resistant to the previous treatments, or that previously undetected acanthamoeba or fungus was present [(Robaei et al., 2018)](https://paperpile.com/c/Vdx3Lu/rQ6P). The option of stopping antimicrobials for a period before sampling to increase the bacterial load may increase the chance of recovering a microbe but it also carries a risk of exacerbation of disease.
	5. **Limited range of licensed antimicrobials for topical use**Treatment of bacterial keratitis is based on topical antimicrobial therapy, with a limited number of licensed options (Table 1). As a result, several antimicrobials that are used to treat bacterial keratitis such as cefuroxime, ceftazidime, vancomycin, teicoplanin and meropenem are used off-label [(Sueke et al., 2010b)](https://paperpile.com/c/Vdx3Lu/UZeN). In the year 2020, 41.5% of prescriptions for topical antimicrobials were for products that were unlicensed for the treatment of microbial keratitis. Frequent topical applications to achieve high initial tissue concentration are recommended [(A. Lin et al., 2019)](https://paperpile.com/c/Vdx3Lu/LhFq), but the optimum frequency is unknown and may vary between patients and with different antimicrobials. New antimicrobials that are active against resistant organisms, or which exploit novel mechanisms of drug action, are usually introduced for systemic use before they are re-purposed for topical delivery. The pharmacokinetics (PK), pharmacodynamics (PD) and toxicity of the antimicrobials are also almost entirely based on systemic delivery with only a limited number of studies relevant to topical therapy. There is a paucity of *in vitro* efficacy data specific to ophthalmic isolates, which is important because rates of resistance may differ between systemic and ocular isolates, and an increase in resistance in isolates from systemic infection may not directly translate to a loss of effect for topically applied antimicrobials. Although there is little evidence that the use of a topical antimicrobial for bacterial keratitis is a strong driver for the the emergence of AMR, antimicrobial stewardship to conserve this precious and finite resource is rarely discussed as a clinical consideration when developing treatment guidelines.
	6. **Unknown relevance of antimicrobial susceptibility to topical therapy**
	The choice of first line therapy for bacterial keratitis requires contemporaneous local data on the spectrum of likely causative bacteria and their susceptibility to available antimicrobials. When the results of susceptibility testing become available the clinician must decide whether to continue or change treatment. To make valid decisions it is important to be able to interpret the results in the context of ocular isolates. The susceptibility data, resistant (R) or susceptible (S), is inferred from the minimum inhibitory concentration (MIC) - the concentration of the antimicrobial that inhibits overnight growth of the bacterium cultured from the patient (refer section 4.2). This MIC is referenced to a breakpoint, which is a chosen concentration (mg/L) of the antimicrobial that defines whether there is a high likelihood of clinical success for that agent against the bacterium. If the measured MIC is significantly less than the breakpoint concentration, the bacterium is usually reported as susceptible to that antimicrobial, and resistant if it is significantly above it. The breakpoint can change with the microorganism, the route of administration, the site of infection or even the clinical indication. However, the antimicrobial MICs measured in the laboratory that define susceptibility have not been validated for bacterial keratitis. The clinical breakpoints of topically applied antimicrobials are unknown, and values based on achievable and safe serum concentrations may not be relevant for topically applied antimicrobials (Figure 1). Although topically applied antimicrobials are delivered frequently and at a high concentration, they may still not be biologically active in the cornea, particularly with continued clearance in the tear film and drainage through the nasolacrimal duct. For example, some glycopeptide antimicrobials such as teicoplanin penetrate the cornea poorly [(Kaye et al., 2009)](https://paperpile.com/c/Vdx3Lu/Bcoz).
	7. **Lack of information to support individualised treatment**
	Treatment of bacterial keratitis with broad spectrum antimicrobials assumes that all isolates of a bacterial species are similar. However, distinct differences derive from their susceptibility to antimicrobials and the virulence factors and toxins that the bacteria produce. The most common isolates from bacterial keratitis, *P. aeruginosa* and *S. aureus,* produce virulence factors. For example, *P. aeruginosa* produces either exotoxin U (ExoU) or S (ExoS). ExoU strains cause lysis of the cell through a phospholipase, while ExoS strains interfere with the cytoskeleton and lead to an invasive infection. We anticipate that in the future the identification of virulence factors that could influence the clinical response in bacterial keratitis will become integral to pharmacological management. Topical antivirulence compounds, such as an exotoxin U inhibitor of *P. aeruginosa*, could be given and or the patient monitored more closely due to the potential for rapid progression of disease. Inherent to individualised treatment is the assumption that knowledge of the identity of the isolate, the susceptibilities, and pathogenicity factors can change outcome by using targeted therapeutics. For this to be a realistic option the test must be accurate and preferably delivered at the point of care. As with current tests, any delay would encourage clinicians to revert to empiric treatment rather than leave the patient untreated pending a result.
2. **THE INVESTIGATION OF BACTERIAL KERATITIS**

A summary of the advantages and disadvantages of the current microbe identification methods used for bacterial keratitis diagnosis is given in Table 2. In practice, investigation for all suspected pathogens (bacteria, acanthamoeba, fungus, virus) can proceed in parallel [(Ung and Chodosh, 2021)](https://paperpile.com/c/Vdx3Lu/kSBs).

* 1. **Clinical signs**
	Clinical signs can be strongly suggestive of bacterial infection, but appearance alone is an unreliable indicator of the type of bacteria causing the infection, or whether there is also a parasitic or fungal infection [(Dahlgren et al., 2007; Dalmon et al., 2012)](https://paperpile.com/c/Vdx3Lu/Tg3qv%2BEXwZ). A consensus assessment by multiple observers may be a more reliable alternative. Deep learning based on corneal images could have a role in determining the domain of the causative organism, such as identification of fungal keratitis, but as yet there are no systems capable of determining the genus [(Kuo et al., 2020)](https://paperpile.com/c/Vdx3Lu/vK92). Currently, the identification of organisms is based on sampling for further investigation, with algorithms to grade the severity of keratitis and the need for specialist care [(Ung et al., 2020; Vital et al., 2007)](https://paperpile.com/c/Vdx3Lu/ltip%2BTDZS).
	2. ***Staining of samples to detect and identify bacteria by microscopy***
	Microscopy is a valuable tool for investigating suspected microbial keratitis, in particular, the early detection of polymicrobial infections involving fungal and acanthamoeba. Samples taken as a scrape from the corneal ulcer are spread or smeared onto a dry glass slide and stained to identify bacteria. The smear can be heat or methanol fixed prior to staining. Gram stain is the most frequently used primary stain for bacteria, with additional stains available if an unusual organism is suspected (e.g. Ziehl-Neelson stain for *Mycobacteria spp*). Both staining and taxonomy require specialist skills and, whilst staining itself is quick and demands little technology, reporting slides can be unreliable and observer dependent [(Samuel et al., 2016)](https://paperpile.com/c/Vdx3Lu/LLLP). Though it cannot offer sensitivities, microscopy may guide initial treatment, especially in situations where there is limited access to a microbiology laboratory. In certain settings it may be appropriate to train ophthalmologists to perform and interpret staining although, even with training, maintaining these skills can be a challenge. The interpretation of stained slides could be supported with artificial intelligence deep learning algorithms (see section 9.5).
		1. ***Is microscopic examination of smears useful?***
		Gram stain, the most frequently used stain, requires minimal resources to differentiate Gram positive and Gram negative bacteria, although this distinction rarely has implications for management if a broad-spectrum antimicrobial is to be used for initial therapy. However, certain microorganisms require immediate attention, such as *Neisseria spp*., to maximise the chances of treatment success, although it can be challenging to definitively distinguish *Neisseria spp.* from other Gram-negative cocci such as *Moraxella sp.*, and thus stains cannot replace culture. If *Mycobacteria spp* infection is suspected, microscopy of a sample stained with Zielhl-Neelson can anticipate the result of culture by several days. Microscopy also offers an opportunity to visualise bacteria, yeasts, filamentous fungi, acanthamoeba, and microsporidia spores that may not have been included in the initial differential diagnosis [(Cury et al., 2018)](https://paperpile.com/c/Vdx3Lu/yFrA). Any advantage of speed will be limited if the sample has to be transferred for expert examination. There is very little published on the potential contribution of a Gram stain to the clinical decision pathway. In a large series of 677 cases described with ulcerative keratitis (where 99% of the isolates were bacteria) the benefit of a Gram stain as an additional test to culture was relatively small. In 6.8% of cases bacteria were identified when culture was negative; conversely in 26.2% the Gram stain was negative when culture was positive [(Asbell and Stenson, 1982)](https://paperpile.com/c/Vdx3Lu/BlGP). Thus a negative Gram stain is not a good indicator of an uninfected corneal ulcer. Drawing further conclusions on the utility of staining for the diagnosis of bacterial keratitis is challenging because a wide range of positive results that have been reported (11.7%–72.5%), with a high proportion of fungal isolates included in some series [(Acharya et al., 2020; Cariello et al., 2011; Fong et al., 2004; Khanal et al., 2005; Leck et al., 2002; Lin et al., 2017)](https://paperpile.com/c/Vdx3Lu/OSvx%2BKu2Y%2BHImv%2B3wCI%2BuO2j%2BRIVV).
		2. ***Can direct bacterial visualisation be improved?***
		Optical molecular imaging using fluorescent probes (Fluorescent SmartProbes) has been used to identify bacteria from suspected bacterial keratitis. The method is based on a fluorophore incorporated into a compound such as 7-nitrobenz-2-oxa-1,3-diazole, a dye that self-quenches in aqueous environments but fluoresces in hydrophobic environments such as the microbial cell membrane. Fluorescent SmartProbes have been shown to be equivalent or better than a Gram stain for detecting Gram-positive and Gram-negative bacteria or fungi within smears from corneal scrapes without the need for sample fixation or washing [(Gunasekaran et al., 2020)](https://paperpile.com/c/Vdx3Lu/D4al). SmartProbes could potentially be incorporated into an impression membrane to allow the direct detection of bacteria without transfer of a sample to a slide. This technique could contribute to treatment decisions at the time of presentation if it could be developed to identify common pathogens such as *Pseudomonas spp.* or *S. aureus*. Fluorescence *in situ* hybridization (FISH) has been reported for the identification of bacteria grown in culture from the primary corneal scrape, but not for identification of bacteria in the primary smear sample [(Patel et al., 2019)](https://paperpile.com/c/Vdx3Lu/OVIj).

		Ocular coherence tomography (OCT) and in vivo confocal microscopy (IVCM) cannot image individual bacterial cells. However, complex aggregates of some bacteria can be seen with IVCM. These include the filamentous figures formed by *Nocardia spp*, and clumps of bacteria or crystalline structures suggestive of *Streptococcus viridans*, *Moraxella spp*, and *Proteus spp* [(Hau et al., 2010)](https://paperpile.com/c/Vdx3Lu/vUYM).
	3. ***Identification of bacteria***
		1. ***Bacterial culture***
		Culture enables bacteria to multiply to sufficient numbers to allow identification and an assessment as to whether growth can be inhibited by ophthalmic-appropriate antimicrobials. The success of primary culture is reduced if there are low numbers of bacteria present in the lesion, inadequate sampling technique, or prior administration of an antimicrobial [(Evaldson et al., 1982; Kugadas and Gadjeva, 2016)](https://paperpile.com/c/Vdx3Lu/3lAT%2BlzeQ). Although it has been considered best practice to inoculate the samples directly onto solid media or into enrichment media, rather than using a transport medium prior to plating, equivalent results are obtained [(Kaye et al., 2003)](https://paperpile.com/c/Vdx3Lu/AGdV). Using a transport culture medium rather than direct plating removes the practical issues associated with collecting multiple samples and using multiple plates in the clinic [(Kaye et al., 2003)](https://paperpile.com/c/Vdx3Lu/AGdV). In addition, there is no difference in isolation rate of samples inoculated directly onto agar plates (blood agar, thioglycolate agar) or into enrichment media (Robertson’s cooked meat, brain heart infusion, blood culture) [(Bhadange et al., 2013; Kaye et al., 2003; Kratz et al., 2006; McLeod et al., 2005)](https://paperpile.com/c/Vdx3Lu/nd7R%2BWH20%2BAGdV%2B7yZK). No comparative economic evaluation has so far been undertaken to compare these methods.

An initial semi-quantitative estimate of the bacterial load can be gained from growth in primary culture, which may indicate the likelihood that it is a pathogen, but the distribution of cultured isolates is skewed by an excess of fast growing virulent bacteria that grow easily on standard media. Colonies growing on solid media away from the point of inoculation are considered to be contaminants. Some uncommon pathogens such as *Mycobacterium spp.* may require selective media (e.g. a Lowenstein-Jensen slope or liquid culture), which should be incorporated as part of the initial investigation if there is a relevant history (e.g. recent LASIK surgery). For all isolates, review of 20 of the largest studies showed a median culture positivity rate of 50.3% (range 32.6–79.4) [(Ung et al., 2019)](https://paperpile.com/c/Vdx3Lu/v7Iw). There is no evidence to suggest a significant contribution for unculturable bacteria in investigation-negative keratitis. Because corneal culture is not a sensitive investigation its utility for routine investigation has been questioned [(McDonnell, 1996; McLeod et al., 1996; Moshirfar et al., 2019)](https://paperpile.com/c/Vdx3Lu/dqke1%2BLhLwW%2BmjnU). Large ulcers are more likely to be culture positive than small ulcers [(Morlet et al., 1999)](https://paperpile.com/c/Vdx3Lu/6TvTa), and recent guidance from the American Academy of Ophthalmology considered the culture of small (<2 mm) peripheral ulcers to be optional as they are less likely than central ulcers to be culture positive, and more likely to heal without visual loss [(A. Lin et al., 2019)](https://paperpile.com/c/Vdx3Lu/LhFq). Apart from cost containment, the rationale for this is unclear as all corneal ulcers begin as small ulcers and it is not known whether there is a difference in bacterial growth phases between early infection in small ulcers and late infection in larger ulcers. The availability of easier methods to collect samples and the use of transport medium means that there are no practical reasons why all ulcers should not be sampled [(Ung et al., 2020)](https://paperpile.com/c/Vdx3Lu/TDZS), which would have the added advantage of providing a more complete picture of the epidemiology of isolates.

Standard laboratory procedures (the type of medium, temperature, duration of culture) are designed to identify expected pathogens. The duration of culture is usually 48 hours, after which slow-growing or fastidious organisms can be overgrown by contaminants. If there are organisms which require more specialist media (*Mycobacteria spp*) or prolonged incubation (*Nocardia spp, Mycobacteria spp*) then it is likely that these will be missed unless specific media, temperatures or duration are added to the investigation. It is therefore important to notify the laboratory if rare (atypical) or unusual (*Nocardia spp, Mycobacteria spp)* bacteria are suspected so that additional plates and extended culture can be considered. Slow growth does not equate to difficulty in culture as long as the correct conditions are provided. It may, however, lead to a delay in initiating the appropriate treatment for slower growing organisms, with an adverse effect on outcome.

* + 1. ***Phenotypic and biochemical testing***
		Identification is relatively routine, but because it can be difficult to discriminate between or within similar species such as CoNS they are often collectively grouped. Phenotypic identification usually follows an algorithmic route following microscopy, e.g. Gram positive cocci are broadly differentiated between staphylococci and streptococci/enterocci using the catalase test, after which *S. aureus* may be identified utilizing tests for coagulase and DNAse, while Beta-haemolytic streptococci differentiated by Lancefield grouping. Another example is that Gram negative bacilli can broadly be differentiated as lactose fermenting or non-fermenting. Non-fermenters are then differentiated by the oxidase test as either order *Enterobacteriales*, or non-enterobacterales, including the pseudomonads. Identification to genus and species level can be undertaken using panels of biochemical tests, e.g. using Analytical Profile Index (API) strips. Improved identification and molecular techniques have changed the classification of suspected pathogens, with the addition of aerobic, non-fermenting Gram-negative rods *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia* [(Lee et al., 2018; Pandita and Murphy, 2011; Sahay et al., 2020; Spierer et al., 2018; Wiley et al., 2012)](https://paperpile.com/c/Vdx3Lu/KKWn%2BwP3n%2BEMab%2BPbZR%2Blvvi). The UK Standards for Microbiology Investigations provides a more detailed description of the steps toward the identification of bacteria [(Public Health England, 2014)](https://paperpile.com/c/Vdx3Lu/wogU).
		2. ***Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS)***A sample from each isolate from the primary culture is placed on a metallic plate and embedded in a laser absorbing matrix [(Alizadeh et al., 2021; Karas and Hillenkamp, 1988; Tanaka et al., 1988)](https://paperpile.com/c/Vdx3Lu/83KA%2BP7kj%2Bf8bN). The matrix is then irradiated with a pulsed laser to trigger ablation and desorption of ionised molecular fragments, which are then accelerated and deflected through a mass spectrometer to a detector. The mass spectra are compared with a library of profiles and, to date, it is possible to identify >1,300 species of Gram-positive, Gram-negative, aerobic and anaerobic bacteria with an accuracy often better than phenotypic and biochemical methods [(Bizzini et al., 2011; Tsuchida et al., 2020)](https://paperpile.com/c/Vdx3Lu/5EhA%2Bbv8t). Although it has reduced the time to identify isolates, a primary culture is still required, and identification is limited to the database of bacterial spectra. In the future, the use of MALDI-TOF MS to identify proteins may enable the identification of bacteria at the strain or serotype level [(Karlsson et al., 2015)](https://paperpile.com/c/Vdx3Lu/4XYl).

		While there has not been a broad species study of MALDI-TOF MS use with ocular isolates, the technique can distinguish *S. pneumoniae* from within the *Streptococcus mitis* group [(Marín et al., 2017)](https://paperpile.com/c/Vdx3Lu/mcNt), and this distinction is important because there are species-specific virulence factors that can affect outcome [(Priya et al., 2014)](https://paperpile.com/c/Vdx3Lu/ygl0). Furthermore, closely related species of CoNS can be separated [(Badenoch et al., 2016; Dupont et al., 2010; McSwiney et al., 2019)](https://paperpile.com/c/Vdx3Lu/cIzT%2BbBqU%2BafqD). Although the option for direct testing of samples from vitreous and blood without primary culture has been reported [(Florio et al., 2018; Song et al., 2017)](https://paperpile.com/c/Vdx3Lu/hehM%2BXF4f), it is likely the bacterial load from corneal samples would be too low as MALDI-TOF MS and similar systems require 104-105 colony forming units (CFU) of bacteria, and there would also be an inability to discriminate polymicrobial samples [(Kok et al., 2011)](https://paperpile.com/c/Vdx3Lu/OAhI). Several studies have investigated MALDI-TOF and molecular methods to detect resistance genes, e.g. MRSA, but detection is not yet reliable enough for clinical use.
		3. ***Polymerase chain reaction (PCR)***
		PCR detects the presence of bacterial deoxyribonucleic acid (DNA) in a primary sample from the ulcer without the need for bacterial growth. A target DNA sequence is amplified with selective primers using a series of cycles (typically 25-35 cycles) of temperature changes for polymerisation.
			1. **Quantitative PCR**Quantitative PCR (qPCR) (or real-time PCR) uses specific primers to target bacterial species. Fluorescent probes allow for real time quantification of the target bacterial DNA during amplification [(Kralik and Ricchi, 2017)](https://paperpile.com/c/Vdx3Lu/YY7J). This contrasts with conventional PCR which identifies whether the targeted DNA is present or absent. In bacterial keratitis, DNA quantification using qPCR has been used to try to distinguish between causative bacteria and ocular flora [(Itahashi et al., 2010; Shimizu et al., 2020)](https://paperpile.com/c/Vdx3Lu/7YklZ%2BLMlx).
			2. ***Multiplex PCR***
			Multiplex PCR is a variant of PCR that allows simultaneous amplification of DNA from several target bacteria by using multiple primers in a single reaction to detect the most likely pathogens. For example, a multiplex assay targeting *Staphylococcus spp.*, *Streptococcus spp.*, *Pseudomonas spp.*, *Moraxella spp*, *Corynebacterium spp.*, *Serratia spp.*, *Haemophilus spp.*, *Enterobacteriaceae spp.* and *Klebsiella spp.* would detect about 85% of the most common corneal isolates. However, rarely isolated, or unsuspected bacteria will not be detected. The potential advantage is rapid identification of a limited range of pathogens and the potential to identify some polymicrobial infections caused by superimposed viral, acanthamoeba or fungal infections. Designing a multiplex assay can be difficult as each primer-target pair requires optimal PCR conditions, which may be optimal for one primer but ineffective for another. Furthermore, the chances of obtaining nonspecific PCR by-products increases with the more primer pairs present in a reaction and these may be amplified more efficiently than the desired target, using up reaction components and further impeding target DNA amplification [(Elnifro et al., 2000)](https://paperpile.com/c/Vdx3Lu/Zzrp).

Itahashi et al demonstrated that a real-time multiplex PCR assay for the simultaneous detection of *S. aureus*, *S. pneumoniae*, *P. aeruginosa*, methicillin-resistant *S. aureus, Fusarium spp* and *Candida spp* could detect and quantitate a presumed causative pathogen in 36 of 40 eyes with a corneal ulcer, but with two bacteria not included in the PCR panel identified by culture [(Itahashi et al., 2010)](https://paperpile.com/c/Vdx3Lu/LMlx). Larger multiplex PCR panels and systems that can detect up to 27 pathogens in 1-2 hours have been developed for the diagnosis of central nervous system infections and pneumonia [(Peri et al., 2021)](https://paperpile.com/c/Vdx3Lu/dhmt).

* + - 1. **Broad-range 16S rDNA PCR**Broad-range 16S rRNA PCR can address the issue of failure to identify unexpected or unusual bacteria when only specific bacteria are targeted [(Patel et al., 2017)](https://paperpile.com/c/Vdx3Lu/LCUJ). The bacterial 16S ribosomal fragment gene (16S rRNA gene) contains highly conserved nucleotide sequences specific for bacteria, interspersed with nine variable regions that are specific for the bacterial genus and species. Identifying bacteria consists of the following stages: a specific region of the 16S rRNA gene is amplified, the PCR product or amplicon is then sequenced, and this sequence is then compared to a reference library. Traditionally, bacterial species were identified using 16S rRNA gene amplification followed by low throughput Sanger sequencing. In recent years, sequencing technologies have significantly evolved with the development of NGS platforms (see section 3.3.5). Similar broad-range PCR primers have been used to detect fungi (18S rDNA and internal transcribed spacer (ITS) genes) and eukaryotes (28S rDNA gene) [(Machida and Knowlton, 2012; Schoch et al., 2012)](https://paperpile.com/c/Vdx3Lu/8bER%2Bxzgf).
			2. **Advantages and limitations of PCR techniques**The proposed advantages of PCR are its speed compared to culture and the potential to identify unusual or slow growing bacteria (*Nocardia spp* and *Mycobacteria spp*), difficult to culture bacteria, and bacteria that are only present in low numbers, such as from patients pretreated with an antibiotic [(Rampini et al., 2011; Rantakokko-Jalava et al., 2000; Saiki et al., 1988)](https://paperpile.com/c/Vdx3Lu/LjJZx%2Bvy0du%2Bjd00). Furthermore, PCR can be targeted to detect bacterial virulence factors (see section 6). Direct PCR performed without the need for DNA extraction and purification has the potential to further reduce bacterial PCR workflow times [(Ben-Amar et al., 2017; Fode-Vaughan et al., 2001; Kai et al., 2019)](https://paperpile.com/c/Vdx3Lu/WCIy%2Bz6jB%2BlshM) and has been successfully performed on ocular surface samples to detect fungus [(Menassa et al., 2010; Zhao et al., 2014)](https://paperpile.com/c/Vdx3Lu/4eYo%2BmBlH). In general medicine, PCR has a role for the identification of bacteria that exist in a dormant form termed the viable but non-culturable (VBNC) state [(L. Li et al., 2014)](https://paperpile.com/c/Vdx3Lu/NkDx). These are living bacteria that could normally behave as a corneal pathogen, but they have lost the ability to grow on standard culture media. Although VBNCs appear to be an extremely rare cause for keratitis, VBNC pathogens should be considered in cases of chronic or relapsing keratitis not responding to treatment.

It is unclear whether any of these advantages of PCR will translate to clinical practice. The time to identification can be similar to culture if laboratory working protocols are considered, *Nocardia spp.* and *Mycobacteria spp.* are rarely reported in most series from temperate countries, and PCR does not routinely provide information on antimicrobial susceptibility. The PCR and Sanger sequencing techniques currently used in clinical practice rely on a predominance of a single bacterial population, meaning polymicrobial infection may only be identified with multiplex PCR. The sensitivity of PCR is not markedly better than culture, with detection rates reported to be between 26%-75%, with a concordance rate between 16S rRNA PCR and culture of only 54%-73% [(Eleinen et al., 2012; Itahashi et al., 2010; Kim et al., 2008; Knox et al., 1998; Rudolph et al., 2004; Somerville et al., 2020a)](https://paperpile.com/c/Vdx3Lu/g6On%2BLVVt%2BdEHd%2BLMlx%2Bakku%2Bd8sE). PCR also does not usually distinguish between live and dead organisms and so has limited use in distinguishing between active infection or inflammation [(Cangelosi and Meschke, 2014)](https://paperpile.com/c/Vdx3Lu/EC6k). Finally, as with culture, PCR can not routinely distinguish between commensals and contaminants from pathogens [(Corless et al., 2000; Eleinen et al., 2012; Itahashi et al., 2010; Kim et al., 2008; Knox et al., 1998; Rudolph et al., 2004; Somerville et al., 2020a)](https://paperpile.com/c/Vdx3Lu/g6On%2BLVVt%2BdEHd%2BLMlx%2Bakku%2Bd8sE%2B1bP9). PCR thus remains a complementary test to culture rather than an alternative. The causes for failure of PCR to identify bacteria are likely to be similar to those of failure for culture, and include an inadequate sample, low DNA load and technical failure of DNA isolation and amplification.

* + 1. **Loop-mediated isothermal amplification (LAMP)**

Loop-mediated isothermal amplification (LAMP) amplifies DNA without needing a series of temperature changes and special equipment, and it is thus less time consuming than conventional PCR [(Notomi et al., 2000)](https://paperpile.com/c/Vdx3Lu/XanB). It has been used to identify a point mutation in a gene that is associated with resistance in the target species, for example *P. aeruginosa* [(Takano et al., 2019)](https://paperpile.com/c/Vdx3Lu/ddEh), although other causes for resistance in the pathogen will be missed. To date, LAMP assays have been developed for the diagnosis of *Acanthamoeba* keratitis [(Ge et al., 2013; Mewara et al., 2017)](https://paperpile.com/c/Vdx3Lu/C5j3%2Bm3uN) but not bacterial keratitis [(Soroka et al., 2021)](https://paperpile.com/c/Vdx3Lu/a0mv).

* + 1. ***Next-generation sequencing for pathogen detection***
		Next generation sequencing (NGS) describes a method to rapidly determine, in parallel, the sequence of a very large number of fragments of DNA or RNA. This is a step beyond targeted PCR because, rather than identifying one or two causative microorganisms, it can characterise whole communities of microorganisms. When applied to bacterial keratitis, NGS could identify whether a change in the ocular surface microbiome from normal (dysbiosis) is a risk for infection. NGS analysis can be target driven (e.g. sequencing of amplified bacterial PCR products to identify species) or non-target driven (e.g. shotgun metagenomics) to identify all microorganisms present. The process traditionally requires nucleic acid extraction, DNA amplification (if necessary), fragmentation and ligation to produce a test library that is sequenced and matched with a database library of reference genome sequences. The basic unit of data is a ‘read’, which is the sequence of a DNA or RNA fragment.

		The principal platforms are short-read (second-generation) sequencing (e.g. Illumina) and long-read (third-generation) sequencing platforms (e.g. Pacific Biosciences, Oxford Nanopore Technologies), which each have advantages and disadvantages in terms of accuracy, throughput and cost. Long-read sequencing platforms overcome some of the genome assembly deficiencies that occur during short reads, however, they require comparatively larger amounts of highly pure input DNA (at least 150ng depending on the sequencing platform, but usually much more to produce optimal sequencing yields), have higher error rates and lower throughputs compared to the short read platforms [(De Coster et al., 2021; Jain et al., 2017; Kilianski et al., 2015; Nicholls et al., 2019)](https://paperpile.com/c/Vdx3Lu/intn%2Bb4N4%2BIgoD%2BaQUZ). The MinION sequencer (Oxford Nanopore Technologies) is a pocket sized device that allows for rapid, real-time long read DNA sequencing and has been successfully used as a diagnostic tool in some settings [(Quick et al., 2017)](https://paperpile.com/c/Vdx3Lu/7U4S).

		Sequences, and thus microorganism taxonomy, can be altered by contamination and bias occurring at each stage of the NGS workflow [(Boers et al., 2019; Browne et al., 2020; Park and Won, 2018; Tan et al., 2019; Witzke et al., 2020)](https://paperpile.com/c/Vdx3Lu/1c22%2BqmMX%2Ba9q3%2B0Cky%2BHDJb). Analysis of the large amounts of information generated means that it is time consuming, labour intensive and expensive because it is reliant on skilled bioinformaticians [(Goldberg et al., 2015; Holmgaard et al., 2020)](https://paperpile.com/c/Vdx3Lu/89oG6%2Bd6Yh). Sequence-based AI has the potential to significantly reduce the time and computational resources required for bioinformatic analysis (see section: 9.5).

			1. ***Targeted amplicon next-generation sequencing (NGS)***
			​​NGS of 16S rRNA gene PCR products is an efficient and relatively inexpensive method (compared to whole genome “shotgun” metagenomics) for determining the relative abundance of all bacteria (in which the 16S gene is present) in a sample, although it will not detect microorganisms that lack a 16S gene (viruses, fungi and protozoa). The 16S rRNA variable region selected for amplification and sequencing depends upon the sample under investigation and the sequencing platform. Short read sequencing platforms are only able to target parts of the 16S rRNA genome, which limits their ability to discriminate between some closely related species [(Noecker et al., 2017)](https://paperpile.com/c/Vdx3Lu/0kxV). Although the V3-V4 hypervariable region is considered to be the best for identifying bacterial diversity, this has not been validated in an ocular surface microbiome [(Klindworth et al., 2013)](https://paperpile.com/c/Vdx3Lu/3nf4).

			Long read sequencing platforms can sequence the entire 16S rRNA gene, improving the sensitivity and accuracy of bacterial identification [(Johnson et al., 2019)](https://paperpile.com/c/Vdx3Lu/EcGU). A study by Kai et al., [(Kai et al., 2019)](https://paperpile.com/c/Vdx3Lu/WCIy) describes rapid bacterial identification using whole 16S rRNA gene amplification and MinION sequencing (see section 3.3.5). This approach has the potential to reduce the time-to-result but has currently only been tested on culture and extracted DNA as opposed to clinical samples (Kai et al., 2019). This study reported that some species were consistently not detected from a simple mock community and that sample handling altered the relative abundance of the community members, with Gram positive bacteria being particularly problematic (Kai et al., 2019). The complexity and low biomass of ocular surface samples means that it is unclear how this method could be applied to suspected bacterial keratitis, and what impact sample processing and amplification would have on keratitis-relevant species.
			2. ***Whole genome ‘shotgun’ metagenomics***In whole genome “shotgun” metagenomics, all the genetic material from a mixed community is sequenced using NGS [(Forbes et al., 2018; Heather and Chain, 2016)](https://paperpile.com/c/Vdx3Lu/RQvC%2BMlXx). All the genomes from bacteria, viruses, acanthamoeba and fungi are sequenced, as well as host human DNA. This has potential for the discovery of new taxa and allows a deeper understanding of the interaction of microorganisms within a community in health and disease. Active microbial genes can be profiled, potentially differentiating colonizing from infectious microorganisms and aiding the detection or discovery of AMR genes in bacterial keratitis [(Břinda et al., 2020; Sagerfors et al., 2021)](https://paperpile.com/c/Vdx3Lu/imis%2BtW6n). Furthermore, human host gene expression may indicate disease risk and cause of infection, and can also be used to monitor host response to infection [(Chiu and Miller, 2019)](https://paperpile.com/c/Vdx3Lu/PlYi). Shotgun metagenomics theoretically provides the diagnostic precision needed for personalized medicine development.
			3. **NGS as a diagnostic tool in bacterial keratitis**In the authors’ experience, pre-amplification DNA yields from corneal impression membrane samples collected from clinically suspected bacterial keratitis patients are typically very low: ranging from 17 - 525ng (unpublished data). It is unknown how much of this represents microbial DNA, but human host cell contamination is likely to be high [(Li et al., 2018)](https://paperpile.com/c/Vdx3Lu/JkYH). The use of NGS as a diagnostic tool for bacterial keratitis is challenging due to the reduction in sensitivity when human host cell contamination is high and microbial yield low. The subtraction of human sequences from large NGS datasets is time consuming and requires significant computational power that is not available in most clinical laboratories. Therefore, until DNA yields from corneal samples can be significantly increased by other means, an amplification step is essential to meet the requirements for current sequencing platforms, but this must be balanced against the risk of amplifying non-relevant DNA.

Several studies have demonstrated the potential of NGS as a diagnostic tool in microbial keratitis. All studies performing NGS on corneal scrape samples needed a DNA amplification step [(Holmgaard et al., 2020; Low et al., 2021; Ren et al., 2021; Seitzman et al., 2019; Shivaji et al., 2021)](https://paperpile.com/c/Vdx3Lu/Pk97%2BHKLC%2B89oG6%2Bki5x%2B1565). Several studies performed V3-V4 16S rRNA gene targeted amplicon NGS [(Holmgaard et al., 2020; Ren et al., 2021; Shivaji et al., 2021)](https://paperpile.com/c/Vdx3Lu/Pk97%2BHKLC%2B89oG6) and one study performed full length 16S rRNA sequencing on two corneal scrape samples [(Low et al., 2021)](https://paperpile.com/c/Vdx3Lu/ki5x). Seitzman et al used amplified DNA and shotgun metagenomics to identify causative microorganisms in five microbial keratitis patients [(Seitzman et al., 2019)](https://paperpile.com/c/Vdx3Lu/1565). Li et al used shotgun metagenomics to identify multiple types of microorganisms from formalin fixed corneas obtained from 16 patients who had keratitis, and compared these with corneas from 4 patients with no clinical or histopathological suspicion of infection. All studies had small sample sizes (between 2 to 200 patients) and used different inclusion criteria (previous antibiotic drop use), NGS workflows and methods for distinguishing between contaminating, commensal, and causative microorganisms (see section 9.4). Seitzman et al subtracted microbe reads identified in samples collected from the patients unaffected eye [(Ren et al., 2021)](https://paperpile.com/c/Vdx3Lu/Pk97). Microorganisms were considered pathogenic if they were known to cause ocular infections and if they represented the most abundant reads in the sample after subtraction analysis. Other studies defined pathogenic microorganisms as those that occupied a predefined amount of the sample [(Holmgaard et al., 2020; Ren et al., 2021)](https://paperpile.com/c/Vdx3Lu/Pk97%2B89oG6). To date, the best approach to distinguishing contaminating and commensal microorganisms using NGS in samples with a low microbial yield such as patients with bacterial keratitis is unknown [(Ozkan and Willcox, 2019)](https://paperpile.com/c/Vdx3Lu/L0Xz).

Therefore, although timescales are reducing and sequencing platforms are becoming more accessible, the high costs, lack of methodology validation, difficulty in interpreting data and the need to be able to analyse and store large amounts of sequence data means that, in the short term, NGS is unlikely to replace conventional diagnostic methods such as culture and PCR in a clinical setting. However, through research, NGS will help identify novel pathogens, virulence factors and AMR genes [(Donkor, 2013; Land et al., 2015)](https://paperpile.com/c/Vdx3Lu/Ejgk%2BY8eT).

1. **RELEVANCE OF CURRENT INVESTIGATION STRATEGIES TO BACTERIAL KERATITIS**
	1. ***Contaminants and investigation-negative keratitis***
	Current techniques do not reliably discriminate between an infected or a sterile corneal ulcer. In published studies a significant proportion of Gram stain (25%-90%) [(Acharya et al., 2020; Cariello et al., 2011; Fong et al., 2004; Khanal et al., 2005; Leck et al., 2002; Lin et al., 2017)](https://paperpile.com/c/Vdx3Lu/OSvx%2BKu2Y%2BHImv%2B3wCI%2BuO2j%2BRIVV) culture (20% - 70%) [(Ung et al., 2019)](https://paperpile.com/c/Vdx3Lu/v7Iw), and PCR results (26%-75%) [(Eleinen et al., 2012; Itahashi et al., 2010; Kim et al., 2008; Knox et al., 1998; Rudolph et al., 2004; Somerville et al., 2020a)](https://paperpile.com/c/Vdx3Lu/g6On%2BLVVt%2BdEHd%2BLMlx%2Bakku%2Bd8sE) are negative and often excluded from clinical trials. In a further proportion the bacteria identified by culture or PCR may not be the pathogen causing disease, so that if CoNS and *Micrococcus* are also excluded the pathogen identification rate by culture may even be as low as 20%. In contrast to the normally sterile environment of samples taken from blood or spinal fluid, samples taken from the ocular surface are exposed to an environment that is not sterile and tests can identify bacteria that are part of the normal ocular surface microbiome as well as pathogens causing infection. Although there is no consensus on the difference between a pathogen and a contaminant there are strategies that attempt to define them. Firstly, a low virulence organism that is only isolated in an enrichment medium may be classed as a suspected contaminant. However, this approach is unreliable because a virulent pathogen such as *P. aeruginosa* may only be isolated from enrichment media if there is a low bacterial load. Secondly, the species or genus of the bacterium may be used as a criterion, such that mixed skin flora are usually grouped as contaminants, although this distinction is mainly based on experience with systemic isolates, and the rule may not be transferable to corneal pathogens. We do not believe that there is a threshold number of colonies on culture that can be used to distinguish a probable contaminant from a pathogen [(Romanowski et al., 2021)](https://paperpile.com/c/Vdx3Lu/MPRf).

	A particular area of uncertainty is the role of CoNS, which are a species group commonly identified in culture and usually considered to be contaminants [(Becker et al., 2014)](https://paperpile.com/c/Vdx3Lu/pBzU). Recent investigations of the population structure of *S. epidermidis* isolates, however, have identified genetic clusters with discrete phenotypic signatures that are proposed to be epidemiological markers that could explain differences in pathogenicity [(Espadinha et al., 2019; Méric et al., 2018)](https://paperpile.com/c/Vdx3Lu/6cNR%2BqEP4). Clinical studies report conflicting results; isolates of *S. epidermidis* from orthopedic device-related infection have identified a correlation between virulence traits and patient outcome [(Post et al., 2017)](https://paperpile.com/c/Vdx3Lu/GgIY), while there was no difference between isolates from prosthetic joint infections and the nares [(Månsson et al., 2021)](https://paperpile.com/c/Vdx3Lu/Wy9X), suggesting that the allocation of all isolates of a CoNS species as potential pathogens is too simplistic. We could not identify any comparable studies for ophthalmology. Several species of the CoNS exhibit accumulations of antimicrobial resistance genes, including *S. epidermidis* (Mansson et al., 2021) and *S. capitis* [(Tevell et al., 2020)](https://paperpile.com/c/Vdx3Lu/o3fh). Collectively, these studies invite further comparisons of curated corneal isolates of *S. epidermidis* and *S. capitis* to compare differences in their genomes and phenotypic signatures and to look for any correlation between these traits and antimicrobial resistance or disease severity, e.g. ulcer size and healing time. Unfortunately, clinical studies that adopt positive culture as their definition for bacterial keratitis rarely describe how they handle cases in which CoNS, or other potential contaminants, are the only isolate. This decision will affect baseline recruitment and epidemiological descriptions of antimicrobial resistance [(Asbell et al., 2020)](https://paperpile.com/c/Vdx3Lu/I5HR). In ophthalmology it is recommended that all organisms should be considered as potential pathogens and reported with their antimicrobial sensitivity if practical. With modern methods, i.e. MALDI-TOF, reporting the identification of all organisms should be a minimum [(Leal et al., 2021; “Public Health England. (2014). Investigation of Intraocular Fluids and Corneal Scrapings. UK Standards for Microbiology Investigations. B 52 Issue 5.2,” 2017)](https://paperpile.com/c/Vdx3Lu/i8Qg%2BCWea).
	2. ***Does systemic administration reflect topical administration***
	To understand the relevance of antimicrobial susceptibility reporting to the treatment of bacterial keratitis, an appreciation of some basic concepts is required [(Callegan et al., 1994b)](https://paperpile.com/c/Vdx3Lu/9g04).
		1. ***Minimum Inhibitory Concentration (MIC) and breakpoint***
		The MIC is the lowest concentration (mg/L) of an antimicrobial that will inhibit the visible growth of a species of bacterium within strictly controlled conditions of incubation time and temperature. The bacteria causing an infection will have a range of MICs and the normal distribution of the MICs in that population, considered with other factors such as dosage, pharmacokinetics, pharmacodynamics, toxicity, and clinical outcomes are then used to establish the threshold concentration (breakpoint), which is used to decide if the bacterial species is susceptible or resistant. Important components of the antimicrobial pharmacokinetics include the half-life, the degree of protein binding, and the time course of the antimicrobial concentration. If the bacterial species have an MIC that is below the breakpoint they are regarded as susceptible (S). Susceptibility indicates that there is a high probability that a bacterial strain is inhibited *in vivo* at the concentration of the antimicrobial that is expected to be achieved at the site of the infection and that there will be a good therapeutic response. If the bacterial species has an MIC above the breakpoint then they would be regarded as resistant (R) with a high likelihood of therapeutic failure. Intermediate (I) often refers to an *in vitro* concentration associated with clinical success with an increased dosage, or it reflects uncertainty from the laboratory perspective when reporting susceptibility or resistance for an observed MIC close to the clinical breakpoint. Knowledge of the previous clinical outcomes with this antimicrobial/bacterial combination is clearly important. For example, if an antimicrobial has a low MIC for a bacterial species, but the clinical outcome was poor, it may be better to select an alternative antimicrobial that has a better clinical outcome despite it having a higher MIC. The advantage of this system of reporting as R or S is that it reduces the complexities of antimicrobial choice to what is essentially a binary decision for the clinician. The major reservation for the ophthalmologist is that the data is based on systemic administration rather than topical treatment, with a paucity of relevant information on clinical outcomes with the chosen antimicrobial/bacterial combinations (Figure 2).

The concentration of the antimicrobials achievable in serum that determines the breakpoint is chosen by regional regulatory bodies such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or The Clinical & Laboratory Standards Institute (CLSI) (USA guidelines), although the values differ between authorities. For topical administration, EUCAST recommends epidemiological cut-off (ECOFF) values (comparison of the antimicrobial MIC distribution representative of a wild type bacterial population with the population resistant to the antimicrobial) to indicate susceptibility. These ECOFF cut-off values serve as a guide and are not breakpoints, and they may vary over time and regions according to recommendations adopted in country or region. Ideally, as further ophthalmic evidence grows, topical ophthalmic breakpoints should be established.

* 1. **Pharmacokinetics of topically applied antimicrobials**The aim of a topical antimicrobial therapy is to achieve a tissue concentration higher than the MIC of the pathogen and lower than the concentration that is toxic for the ocular surface. The ability of the antimicrobial to achieve and maintain the MIC, or higher, even in the deeper corneal layers will determine its ability to eradicate an infection. Pharmacokinetics (PK) determines the optimum dosage regimen (how much and how often) required to maintain the concentration of an antimicrobial in the cornea within the therapeutic range. Pharmacodynamics (PD) describes the intensity of the antibiotic effect in relation to its tissue concentration. The parameters that characterize topical antimicrobial PK are the corneal penetration to the anterior chamber, clearance from the eye, the maximum concentration (Cmax), and the time that the concentration of the antimicrobial remains above the MIC (Figure 3). The area under the curve (AUC), which is derived from the height (concentration) and the duration (time) that the antibiotic remains above the target MIC following a single dose, is a measure of total effective antibiotic exposure. The AUC is a useful parameter to compare whether different formulations (e.g. preserved or unpreserved, ointment or drop, topical or subconjunctival) result in an equivalent exposure of the tissue to the antibiotic. Corneal penetration and the concentrations achieved in the aqueous depends on several antimicrobial-specific factors such as the structure and molecular weight of the antibiotic e.g. ciprofloxacin (MW 331.3) or vancomycin (MW 1449.3), and the time the solution remains in contact with the cornea e.g. viscosity of an ointment, pH and lipophilicity. Optimal dosing depends on the susceptibility of the bacteria, and the PK of the antibiotic (Figures 3 and 4). There is an assumption that there is little variability in PD between patients; however, patient-specific factors, including the size of an epithelial defect or ocular surface disease can affect the PD; a watering eye, discharge, nasolacrimal duct obstruction or a keratinised ocular surface, will all affect the PK.

	Compared to data on the systemic administered antimicrobials, there is relatively little information on the PK or PD of topically administered antimicrobials in the cornea. For cases of keratitis involving the posterior layers of cornea, it is important to consider the aqueous as well as the corneal concentrations (Figures 4 and 5). information on the corneal concentrations of antimicrobials licensed for the treatment of microbial keratitis is available from the manufacturer. For all these reasons, it is an oversimplification to assume that moxifloxacin 0.5%, which delivers 5 g/L of antimicrobial directly to the corneal surface, will achieve a tissue concentration and biological activity far in excess the MIC of most pathogens (e.g. MIC of *P. aeruginosa* 0.5 g/L) (Figures 5 and 6). Different antimicrobials will have inherently different MICs to bacteria. Ideally the antimicrobial with the lowest MIC and an expectation to achieve a concentration above the MIC in the cornea and aqueous should be selected. For example, for *P. aeruginosa*, ciprofloxacin has a lower MIC compared to ofloxacin, levofloxacin and moxifloxacin (Figure 7).
	2. **Interpreting the response of bacterial keratitis to an antimicrobial**
	To determine ophthalmic breakpoint concentrations would require knowledge of the relationship between the MIC of the topically applied antibiotic and the clinical outcome, usually measured as time to corneal re-epithelialization, size and density of scar, or spectacle corrected visual acuity. Ulcer sizes vary and to try and standardise these outcomes, the ratio of the healing time to ulcer size has been proposed (Figure 2). Several studies have shown that there is such a relationship [(Chen et al., 2008; Kaye et al., 2010; Lalitha et al., 2012a; Wilhelmus et al., 2003)](https://paperpile.com/c/Vdx3Lu/oqb3%2BdrLL%2BouhS%2B8SEzj). In particular, we and others have shown that for *Pseudomonas* spp. *S. aureus* and *Enterobacteriaceae* there is a linear association between the MIC of a fluoroquinolone and time to epithelial healing [(Kaye et al., 2010)](https://paperpile.com/c/Vdx3Lu/oqb3). In contrast, for bacteria with questionable pathogenicity such as *Streptococcus* *spp*. and CoNS there was not a significant association, although it is unknown whether this reflects the limited activity of the fluoroquinolones against *Streptococcus* *spp*. It would be appropriate to repeat this study with chloramphenicol, which has excellent corneal penetration to the anterior chamber, as well as good activity against *Streptococcus* spp., or with a glycopeptide such as vancomycin or teicoplanin, providing there is an epithelial defect, as they only have poor penetration across an intact epithelium (Kaye et al., 2009). Until ophthalmic breakpoints have been established, we believe that a goal should be to provide the ophthalmologist with the MIC of an isolate to a range of eye-appropriate antimicrobials, in addition to whether the bacteria is susceptible or resistant using a systemic break point. For centres using disc diffusion this may entail a change in laboratory practices to gradient diffusion testing, although performing manual MIC determination for many agents in a polymicrobial infection may be impractical. For centres using automated susceptibility testing (AST) platforms such as BD Phoenix or Vitek this is achievable. Providing the MIC has immediate and distinct advantages. For example, if the MIC is close to the reported concentrations of antimicrobials achieved in the cornea, the clinician has the option of increasing the dosage (e.g. frequency of application, intrastromal injection), changing the antimicrobial or adding in a further antimicrobial to achieve an additive or synergistic response.
1. **BACTERIAL SUSCEPTIBILITY TO ANTIMICROBIALS**
	1. **Methods for determining antimicrobial MIC**
	There are several *in vitro* methods to measure MICs [(Khan et al., 2019)](https://paperpile.com/c/Vdx3Lu/OoeK), but there is currently no clinically available and validated molecular method to determine the antimicrobial sensitivity of the common ocular pathogens. For sensitivity testing, a suspension is prepared from several morphologically similar colonies selected from the primary culture, which will have a range of MICs [(Kowalska-Krochmal and Dudek-Wicher, 2021)](https://paperpile.com/c/Vdx3Lu/qBhP).
		1. ***Disk diffusion***
		This uses filter paper disks impregnated with a known amount of antimicrobial and placed on an agar plate that has previously been seeded with a surface lawn of the bacterial isolate (Figure 1). The test antimicrobial diffuses through the agar, with a logarithmic reduction in concentration over distance, creating a gradient of reducing concentration around the disks. As the surrounding bacteria grow there is a zone of inhibition (ZOI), with a diameter related to the molecular size, charge and MIC of the antimicrobial. Disk diffusion breakpoints are then interpreted from the ZOI using published standards. The method is not suited to all antimicrobial/bacterial combinations, such as susceptibility testing of *S. aureus* with the large molecular weight glycopeptides vancomycin and teicoplanin, when a gradient diffusion test is performed. Colistin is also unsuited to disk diffusion and requires broth tube dilution to measure the MI. There is also limited reference data for many bacteria (strains of *Pseudomonas*, *Bacillus* and *Corynebacterium*) and the performance is poor when analyzing slow-growing and fastidious bacteria.
		2. ***Tube dilution***
		Antimicrobial concentrations are logarithmically diluted (two-fold dilutions) in tubes containing bacteria in a nutrient broth dilution and incubated overnight (Figure 8). The turbidity, or the fluorescence spectra of the medium, is then measured and used to assess bacterial growth. A clear medium indicates no bacterial growth and the concentration of the antimicrobial in that tube is the MIC. Pre-filled disposable cassettes are used in automated screening machines (e.g. BD Phoenix). Although these instruments measure the MIC, inbuilt software compares this to a reference breakpoint and the output is an S or R value. Because the database of breakpoints are based on treatment for systemic disease, some first-line ophthalmic treatments may not be available, such as moxifloxacin susceptibility in *Pseudomonas spp*., because moxifloxacin is rarely used systemically for *Pseudomonas* infection.
		3. ***Gradient diffusion (Etest)***
		The Epsilometer (Etest) uses a reducing gradient of antibiotic concentration coated onto plastic strips that are then placed onto bacteria spread on a solid medium. Elliptical inhibition zones appear around the strips, with the MIC indicated at the intersection of the inhibition zone and the strip edge (Figure 9). It is cheap and simple to use and interpret. Strips with two different antibiotics placed at right-angles can then be used to assess antimicrobial combinations for synergy, addition, indifference and antagonism [(Sueke et al., 2010a)](https://paperpile.com/c/Vdx3Lu/rQdb).
		4. ***Limits of planktonic investigation in the presence of biofilm***
		Antimicrobial testing is usually performed with bacteria grown in a planktonic mode (grown in suspension in a liquid). However, some corneal pathogens can form a biofilm that can reduce their susceptibility to antimicrobials. *Nocardia spp.,* which is a common cause for bacterial keratitis in South Asia [(Srinivasan et al., 2012)](https://paperpile.com/c/Vdx3Lu/wbEK), typically requires prolonged antimicrobial treatment with a tendency for recurrence, possibly due to the formation of biofilm [(Faria et al., 2015)](https://paperpile.com/c/Vdx3Lu/5giQ). *P. aeruginosa* may be protected from contact lens disinfection by biofilm, and *S. viridans* can form a biofilm when the inflammatory response is suppressed by topical corticosteroid (crystalline keratopathy). It is likely that the MIC of an antimicrobial against *P. aeruginosa* would be substantially higher if it were tested after the formation of biofilm compared to testing in suspension [(Smith et al., 2020)](https://paperpile.com/c/Vdx3Lu/yjgL).
	2. **Antimicrobial resistance (AMR) and bacterial keratitis**
	Antimicrobial resistance (AMR) is an intrinsic or acquired characteristic encoded by genes that can be transferred between bacteria [(Blair et al., 2015)](https://paperpile.com/c/Vdx3Lu/0eIt). The mechanisms of AMR include prevention of access, modification, or inactivation of the antimicrobial [(R. B. Singh et al., 2021)](https://paperpile.com/c/Vdx3Lu/iIaE). AMR has made some systemic bacterial infections difficult to treat and an increase in AMR is a global phenomenon [(Alexandrakis et al., 2000; Leibovitch et al., 2005; Littmann and Viens, 2015; Shalchi et al., 2011)](https://paperpile.com/c/Vdx3Lu/vjOs%2BiLVx%2BV8Nx%2B8qte). However, although an increase in AMR in isolates from bacterial keratitis has been documented, it is not as apparent as in non-ocular infections, and the impact of this on outcomes has not been fully assessed. The AMR of a bacterium is determined by a combination of intrinsic characteristics, such as the permeability of the cell membrane, and specific AMR genes. Interestingly, the presence of AMR genes may predate the selection pressures from the clinical use of antimicrobials; genes associated with resistance to β-lactam, tetracycline and the complete vancomycin resistance element VanA have been identified in bacteria recovered from ancient permafrost [(D’Costa et al., 2011)](https://paperpile.com/c/Vdx3Lu/5g7Q). AMR can occur through increased expression of an existing AMR gene or by gaining new genetic material. This process of gaining new genes is facilitated in bacteria by horizontal (lateral) gene transfer, transduction via phages, and conjugation of AMR-encoding plasmids [(Tenover, 2006)](https://paperpile.com/c/Vdx3Lu/yQ5Y). Resistance mechanisms include altering the amount of antibiotic that gets into the bacterial cell (eg. through reduced porins), modification of antibiotic targets, efflux pumps that pump antibiotics out of the cell, and neutralizing the antibiotic by enzymatic action.

	An increase in the prevalence of resistance to all classes of the fluoroquinolones has been reported in ophthalmic isolates [(Alexandrakis et al., 2000; Garg et al., 1999; Goldstein et al., 1999; Lalitha et al., 2017; Ni et al., 2015; Peng et al., 2018; Ray et al., 2013)](https://paperpile.com/c/Vdx3Lu/V8Nx%2B0Wzmk%2B4SQc%2BicwY%2BFx1f%2Br65Jx%2Biwa7). However, it is again important to note in this context that the definition used to determine resistance is based on systemic breakpoints, and the phenomenon does not necessarily correlate with a change in clinical response when treating keratitis. Although *P. aeruginosa* susceptibility to either ciprofloxacin or moxifloxacin is still approximately 80% worldwide [(Kaliamurthy et al., 2013; Lalitha et al., 2017; Lichtinger et al., 2012; Ni et al., 2015; Peng et al., 2018)](https://paperpile.com/c/Vdx3Lu/qbml%2BFx1f%2BZyNN%2Biwa7%2Br65Jx), there are several studies that have highlighted the presence of high proportions of AMR bacteria in ocular infections, particularly in the US [(Asbell et al., 2020)](https://paperpile.com/c/Vdx3Lu/I5HR), China [(L. Lin et al., 2019)](https://paperpile.com/c/Vdx3Lu/inMP), and India [(Singh et al., 2020)](https://paperpile.com/c/Vdx3Lu/UtUY). In Southern India, there was a particularly sharp increase in resistance of *P. aeruginosa* to moxifloxacin, from 19% (95% CI 5.4% to 41.9%) in 2007 to 52% (95% CI 29.8% to 74.3%) in 2009 (P=0.024) [(Oldenburg et al., 2013)](https://paperpile.com/c/Vdx3Lu/dLae). Two separate 20-year reviews found increasing methicillin-resistant *S. aureus* keratitis from 1993 to 2015 [(Chang et al., 2015; Peng et al., 2018)](https://paperpile.com/c/Vdx3Lu/798v%2Br65Jx). However, without reporting the MICs it is difficult to interpret these findings, and the level of AMR may represent the prevalence in the community, with consequent contamination of the ocular surface [(Blomquist, 2006)](https://paperpile.com/c/Vdx3Lu/G7ns), rather than the selection of resistance by ophthalmic prescribing practices. In general, high level resistance is less likely to develop for an antimicrobial that acts on more than one bacterial site, e.g. a fluoroquinolone such as moxifloxacin that acts on both bacterial gyrase and topoisomerase IV, compared to ciprofloxacin acts predominantly on gyrase [(Aldred et al., 2014; Spence and Towner, 2003)](https://paperpile.com/c/Vdx3Lu/DNPE%2BakUQ). However, the primary antimicrobial target can also vary between bacterial species [(Khodursky et al., 1995; Pan et al., 1996)](https://paperpile.com/c/Vdx3Lu/Erm2%2B0fVx). Overuse, misuse, and the indiscriminate use of antibiotics in agriculture are thought to be the key drivers for the emergence and selection of AMR (Martinez et al., 2012). Many classes of antimicrobials are given to animals. Moxifloxacin is licensed for veterinary use and Ciprofloxacin is licensed to treat animals that are used for human food production. If there is no waste containment ciprofloxacin residues can then persist in the soil [(Riaz et al., 2018)](https://paperpile.com/c/Vdx3Lu/hjLr). Unfortunately, simply withdrawing an antimicrobial from general use does not mean that the levels of resistance in the community will return to the levels of the pre-prescription era [(Enne et al., 2001; Sundqvist et al., 2010)](https://paperpile.com/c/Vdx3Lu/3O9A%2B8dZ0).

	Fluoroquinolone-resistance, methicillin-resistance and multidrug resistant (MDR) bacteria, defined as an acquired resistance to at least 1 agent in 3 or more antimicrobial classes, has also been reported in isolates from bacterial keratitis [(Acharya et al., 2020; Cariello et al., 2011; Hernandez-Camarena et al., 2015; Kaliamurthy et al., 2013; Vola et al., 2013)](https://paperpile.com/c/Vdx3Lu/OSvx%2BRIVV%2Bqbml%2B7eWd%2BctOS). In Southern India MDR has been reported in *S. pneumoniae* (44%), *S.epidermidi*s (14.8%), *S. aureus* (14%), and *P. aeruginosa* (6%). Some common ophthalmic pathogens, particularly *P. aeruginosa*, can show MDR, [(Magiorakos et al., 2012; Tacconelli et al., 2018)](https://paperpile.com/c/Vdx3Lu/0mST%2B1LVX), with MDR *P. aeruginosa* emerging as a cause of bacterial keratitis in South Asia [(Fernandes et al., 2016; Vazirani et al., 2015)](https://paperpile.com/c/Vdx3Lu/j36a%2Bp0YV). In South China an increase in MDR in Gram-positive cocci was reported between 2010 to 2018, while the susceptibility of Gram-negative bacilli to fluoroquinolones and aminoglycosides was stable [(L. Lin et al., 2019)](https://paperpile.com/c/Vdx3Lu/inMP). Inevitably there have been reports of MDR *P. aeruginosa* requiring treatment with antibiotics of ‘last resort’ such as topical colistin and carbapenems [(Chatterjee and Agrawal, 2016; Vazirani et al., 2015)](https://paperpile.com/c/Vdx3Lu/DbeA%2Bp0YV).
	3. ***Limits of bacterial surveillance***The benefit of following the 'ecology' or spectrum of bacterial infections and their sensitivity to antimicrobials is to ensure that empiric treatment guidelines are still appropriate. A potential problem with extended audits is that the results could be skewed by changes in the methods of identification and the methodology and definition of breakpoints. For example, there was a significant change around 2014 when antimicrobial susceptibility testing methodology in the UK changed from the established British Society of Antimicrobial Chemotherapy methods to those set by the EUCAST. Subsequently, in 2020 EUCAST introduced a change in the way the laboratory reported susceptibilities, replacing 'Intermediate (I), (a composite classification combining the possibility of laboratory uncertainty and possibility of clinical success with higher dosing), with two new categories; 'Susceptible, Increased exposure’ (I), (where exposure refers to the amount of antimicrobial that reaches the microorganism either through increased dosing or concentration at the site of infection), and a new classification of Area of Technical Uncertainty (ATU). An antimicrobial reported ‘I’ to a microorganism under post-2020 EUCAST criteria implies a likelihood of clinical success if a higher-dosing regime is used or if the antimicrobial is concentrated at the site of infection, e.g. when topical antimicrobials are used.

	Overall, it is clear that AMR is a threat to current treatment practices across many bacterial infections. These increases would require either higher doses of existing antimicrobials (an increase in MIC does not mean the antimicrobial is no longer effective) or alternative strategies. Whereas for systemic use there are toxicity issues that limit the concentrations used, toxicity is not as problematic when considering topical treatment of the eye. Thus, the need for ‘increased exposure,' i.e. the option to use a higher dose, may be an acceptable strategy for topical antimicrobials.
	4. ***Drivers for emerging resistance in ophthalmic isolates***
	It is unclear whether topical treatment with antimicrobials is a significant driver for the emergence of antibiotic resistance on the ocular surface, or whether resistant bacteria can disseminate from the ocular surface into the community. It is informative that despite availability of chloramphenicol drops over the counter in the UK since 2005, and the absence of its use for other indications, there has been no evidence of increasing resistance in conjunctival isolates over a 12 year period from 2001 to 2012 [(Silvester et al., 2017; Walker et al., 1998)](https://paperpile.com/c/Vdx3Lu/R6Ld%2BcW97). A study of over 3200 ocular isolates collected in the USA from 2009 to 2018 found meticillin resistance in 34.9% of S. *aureus* isolates*,* with a high concurrent resistance to fluoroquinolone; however, there was not an increased overall resistance during the study period [(Asbell et al., 2020)](https://paperpile.com/c/Vdx3Lu/I5HR). In the UK over a 10 year period, methicillin resistance among *S. aureus* was 8.3%. In this study, a comparison of clinical outcomes in cases of *S. aureus* MRSA and MSSA keratitis showed the main determinant of a poor outcome was not the presence of MRSA but the presence of lukSF-PV amongst MSSA isolates [(Sueke et al., 2013)](https://paperpile.com/c/Vdx3Lu/LXss), demonstrating the importance of bacterial virulence factors in addition to antimicrobial susceptibility in determining outcomes As opposed to the systemic use of antimicrobials, where unintended sites are exposed to the antimicrobial that can promote resistance, the bacteria exposed to topical antimicrobials are mostly constrained to bacteria on the ocular surface and in the nasolacrimal duct. This limits exposure of the antimicrobial to environmental and systemic bacteria with less risk of disseminating resistance.
	5. **Strategies to reduce pressure to select resistant strains**
	The development of resistance is affected by the MIC and mutation frequency of the causative bacteria, such that a key strategy to minimizing AMR is to achieve optimal drug exposure. The probability of AMR significantly increases if bacteria are exposed to an antimicrobial at a concentration that is less than the MIC. The more susceptible the strain is to the antibiotic, the greater the likelihood that its MIC will be below the ECOFF and that treatment will not select a drug-resistant subpopulation. With inadequate treatment the apparent MIC of the residual bacterial population will increase, as will the risk of treatment failure [(Andersson et al., 2019)](https://paperpile.com/c/Vdx3Lu/rBSp). Mechanical factors, such as a biofilm associated with a contact lens, or corneal glue used for a corneal perforation, will affect the pharmacodynamics of an antimicrobial and increase the risk of AMR. It is against this background that combination treatment has a potential role. The AUC/MIC ratios of combination therapies may be additive and, particularly if they have different modes of action with no additional toxicity, they can reduce the emergence rate of bacterial resistance. Lower rates of resistance for various pathogens with combination versus monotherapy regimens have been demonstrated [(Shankar et al., 2012)](https://paperpile.com/c/Vdx3Lu/DggO).
2. **BACTERIAL DETERMINANTS OF OUTCOME**Two of the most common isolates from bacterial keratitis are *P. aeruginosa* and *S. aureus*. Both bacteria can express virulence factors, which raises the important question as to whether it is possible to rapidly identify bacterial virulence or toxin production at the point of first care and modify management accordingly. It has been shown that keratitis isolates differ from non-keratitis isolates and, importantly, have a differential expression of virulence factors. For example, *P. aeruginosa* keratitis isolates cluster genotypically, are more mobile and have a high proportion of exotoxin U (Figure 10).
	1. ***Virulence factors of P. aeruginosa***Exotoxins can cause rapid and irreversible damage to the cornea from the onset of infection and they can facilitate intracellular bacterial survival, which increases the risk of treatment failure. This is a key challenge for novel therapies [(Sewell et al., 2014)](https://paperpile.com/c/Vdx3Lu/21Qi).
		1. ***The type 3 secretion system (T3SS) of P. aeruginosa***
		Interaction between the bacterium and the corneal epithelium stimulates assembly of a needle-like apparatus called the T3SS. Upon contact with a target cell, a translocation pore forms across the host cell membrane, allowing the T3SS injectosome to actively secrete exotoxin virulence factors into the host cytoplasm. The components of the mechanism are illustrated in Figure 11. Certain virulence factors have distinctive roles and facilitate tissue destruction, manipulate target host cell signalling pathways and/or subvert the host immune responses. There are four distinct Exotoxins: ExoU, ExoS, ExoT and ExoY. Clinical isolates, however, possess either ExoS or ExoU, but very rarely both, with a high proportion of ExoU in *P. aeruginosa* isolated from corneal ulcers [(Shankar et al., 2012)](https://paperpile.com/c/Vdx3Lu/DggO). ExoS expression has been associated with endocytic uptake and intracellular survival of bacteria in chronic infections, ExoU expression is associated with aggressive acute infections due to phospholipase activity that destroys the plasma membrane of the host cell. In keratitis, isolates of *P. aeruginosa* that possess ExoU are associated with more severe disease. ExoU is also vital to the survival of *P. aeruginosa* during the early stages of infection. Delays in ExoU expression leads to reduced bacterial burdens in the lungs of mice [(Howell et al., 2013)](https://paperpile.com/c/Vdx3Lu/DrgV), which is suggested to be because ExoU can be targeted to infiltrating neutrophils, thus subverting *P. aeruginosa* clearance by the innate immune system.
		**ExoU**
		Although the mechanisms of ExoU regulation are not fully understood, ubiquitin-binding to the plasma membrane and oligomerization followed by phospholipase A2 activity have been described. In the bacterium, ExoU binds to the *P. aeruginosa* chaperone SpcU and is transported to the T3SS machinery where it is secreted into the target host cell by the action of ATPases. Once inside the host cell cytoplasm, ExoU interacts with host co-factors ubiquitin, the chaperone cysteine string protein (CSP), and phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane. The 4-helical bundle domain, in the C-terminus of ExoU, is responsible for its insertion into the plasma membrane, where ExoU is found to form multimers and exert catalytic activity, causing cell lysis (Figure 11).

		The phospholipase activity of ExoU directly degrades target cell plasma membranes, causing acute tissue damage and the release of arachidonic acid, which exacerbates the inflammatory immune response. The catalytic activity of ExoU also increases IL-8 synthesis in host tissue as a result of JNK/MAPK pathway activation, encouraging the recruitment of neutrophils that mediate further tissue damage and subvert the innate immune response by increasing epithelial cell permeability and, as a consequence, potentiation of bacterial penetration.

		**ExoS**
		ExoS has a N-terminal GTPase-activating protein domain (GAP) and a C-terminal ADP ribosyltransferase (ADPR) domain that interferes with the target host cell cytoskeletal function, impairing adhesion and cell migration [(Kroken et al., 2018; Vareechon et al., 2017)](https://paperpile.com/c/Vdx3Lu/L9cp%2BAKxy). The GAP domain targets a subset of human GTPases, which leads to remodelling of the host actin cytoskeleton. The ADPR domain leads to disruption of actin polymers and their anchorage with adhesions leading to cell rounding. Stains of *P. aeruginosa* that express ExoS propagate their pathogenicity by becoming internalised by host cells where they continue to secrete ExoS. Once internalised, the ADPR activity of ExoS allows the *P. aeruginosa* to generate membrane bleb niches, where they are protected from lysosomal degradation [(Kroken et al., 2018)](https://paperpile.com/c/Vdx3Lu/L9cp). Although the mechanisms have yet to be fully explored, interactions with endogenous human proteins, such as the 14-3-3 protein regulators of cell signaling, are a requisite for ExoS stabilisation and activation in host cells.
		2. ***Inhibitors of the T3SS machinery***The cyclic peptomers, EpD-1,2,4′N and 4EpDN, specifically block type III secretion in *P. aeruginosa* [(Lam et al., 2017, 2021)](https://paperpile.com/c/Vdx3Lu/3ZwK%2B2wXW). Although the mechanism is unclear, these peptomers appear to inhibit assembly of the T3SS injectasome. They have no observable toxicity in HeLa cells, which suggests they could be used at high concentrations to achieve a therapeutic dose when applied topically. Hydroxyquinolines inhibit the ATPase activity required for T3SS effectors to be unfolded and translocated through the injectasome needle apparatus. The hydroxyquinolone INP1855 was found to protect eukaryotic cells from *P. aeruginosa* T3SS mediated cytotoxicity and in an in vivo mouse model of lung infection, it reduced lung injury and bacterial dissemination, with improved survival [(Anantharajah et al., 2016)](https://paperpile.com/c/Vdx3Lu/xWIq). Monoclonal antibodies that target PcrV can inhibit T3SS mediated toxicity in animals infected with clinical strains of *P. aeruginosa, reduce* bacterial load and reduce the inflammatory immune responses [(Ranjbar et al., 2019)](https://paperpile.com/c/Vdx3Lu/6uF8). As a result, several promising anti-PcrV therapeutic antibodies have recently entered Phase I and II clinical trials.

			1. ***Therapeutic prospects that target ExoS***Because the catalytic activity of the ADPR domain is pivotal for bacterial dissemination in *P. aeruginosa* that express ExoS, targeting ExoS ADP-ribosyltransferase activity may be a viable therapeutic strategy. Exosin, Diosmin, 4296-1011, Everninic acid and E216-5303 were among a panel of the most promising ExoS inhibitors that could prevent recombinant ExoS mediated cytotoxicity in yeast. Through analysis of their effects on ExoS ADPR activity *in vitro*, these compounds were shown to be competitive inhibitors, preventing ExoS mediated ADP-ribosylation in human cells [(Arnoldo et al., 2008)](https://paperpile.com/c/Vdx3Lu/bp14), which led to the development of a more potent ExoS inhibitor, exosin-5138 (12 IC50 µM), which could protect Chinese Hamster Ovary (CHO) cells from ExoS mediated toxicity, even at late stages of infection..

			Recent evidence suggests that the hydrophobic interface between 14-3-3 and ExoS, is a good target site for small chemical inhibitors. STO1704, which is a small molecule that at the rim of the hydrophobic ExoS- binding site is able to inhibit ADP-ribosylation by ExoS. Although high concentrations of STO1704 were required to achieve these effects (IC50 of 638 μM for ExoS and 726 μM for ExoT), this proof of concept supports the idea that compounds could be developed that inhibit protein to protein interactions and inhibit ExoS.
			2. ***Therapeutic prospects of targeting of ExoU***Neutralising ExoU with small molecule inhibitors is an attractive therapeutic approach to reduce proinflammatory signaling. A panel of ExoU selective inhibitors, including pseudolipasin A, have been identified following high-throughput screening to detect increases in cell viability after infection with ExoU expressing *P. aeruginosa* [(Howell et al., 2013)](https://paperpile.com/c/Vdx3Lu/DrgV). These compounds inhibited recombinant ExoU in vitro and also protected CHO cells from ExoU expressing *P. aeruginosa* at micromolar concentrations of the inhibitor. In an *in vitro* scratch assay, treatment of *P. aeruginosa* that express exotoxin U with a fluoroquinolone at a concentration below the MIC and with an ExoU inhibitor leads to better corneal epithelial cell survival and healing despite persistence of *P. aeruginosa* (Figure 12) [(Foulkes et al., 2021)](https://paperpile.com/c/Vdx3Lu/BMPD).

			The multiple dynamic conformational changes that ExoU adopts may be targetable using structure guided design to produce small molecules that attenuate ExoU activity. Compounds that prevent PPIs with obligatory host activating cofactors and chaperones such as ubiquitin and CSP, that prevent ExoU oligomerization and localisation to the plasma membrane or bind to the catalytic domain of ExoU would be candidates. The mechanisms by which currently ExoU inhibitors prevent catalytic activity are not yet understood. This highlights the need for ExoU-compound co-crystal structures and the development of biochemical assays to interrogate how compounds inhibit ExoU.
	2. ***Staphylococcus aureus* virulence***S. aureus* is a well-equipped opportunistic pathogen with a wide array of secreted virulence factors. However, only a limited number of these factors have been investigated for their role in ocular colonisation and disease (Figure 13).
		1. **Alpha toxin**The role of this haemolysin in the pathogenesis of ocular disease is well understood [(Callegan et al., 1994a; McCormick et al., 2009; O’Callaghan et al., 1997)](https://paperpile.com/c/Vdx3Lu/98Un%2BJzWP%2BfHun). The enzyme mediates bacterial invasion and, more recently, it was shown to delay wound closure [(Putra et al., 2019)](https://paperpile.com/c/Vdx3Lu/dgq7). Alpha toxin is a beta-barrel pore forming toxin that binds as a monomer to various receptors on eukaryotic cells that depend on cell type. At high concentrations the monomer also binds to the cell membrane lipids, sphingomyelin and phosphatidylcholine [(Hildebrand et al., 1991)](https://paperpile.com/c/Vdx3Lu/bqmq). Monomers accumulate by interaction to form a heptameric pore that is permeable for cations (Na+, K+ or Ca2+) and small molecules (ATP) and causes changes in membrane potential, proton motive force and disrupts cellular processes [(Bhakdi and Tranum-Jensen, 1991)](https://paperpile.com/c/Vdx3Lu/6AyG). At lower concentrations, alpha toxin leads to a disruption of focal adhesions and tissue barriers [(Wilke and Bubeck Wardenburg, 2010)](https://paperpile.com/c/Vdx3Lu/vnfa), At increased concentrations alpha toxin leads to cell lysis.

		Gene inactivation of *hla* encoding the haemolysin in different *S. aureus* strains significantly reduces corneal epithelial damage and improves wound healing [(Callegan et al., 1994a; Dajcs et al., 2002; Putra et al., 2019)](https://paperpile.com/c/Vdx3Lu/98Un%2Bszfy%2Bdgq7); supporting the concept of alpha toxin inhibition as a therapeutic strategy. Studies have also reported successful inhibition of alpha toxin. A combination of cyclodextrin and cholesterol inhibits the activity of haemolysin *in vitro* and *in vivo* [(McCormick et al., 2009)](https://paperpile.com/c/Vdx3Lu/JzWP), which is likely to be a consequence of alpha toxin-ADAM10 complex assembly in cholesterol/sphingolipid-rich caveolar rafts [(Wilke and Bubeck Wardenburg, 2010)](https://paperpile.com/c/Vdx3Lu/vnfa). Ikemoto et al., (2020) have demonstrated that the antibiotic azithromycin inhibits toxin production in addition to its antibiotic and anti-inflammatory effects, which reduces ocular surface damage in experimental *S. aureus* keratitis [(Ikemoto et al., 2020)](https://paperpile.com/c/Vdx3Lu/fOmW). Linezolid also limits *in vivo* toxin production, including alpha toxin [(Diep et al., 2013)](https://paperpile.com/c/Vdx3Lu/bwSv). A better understanding of the persistence of alpha toxin and half-life of the enzyme in corneal tissue will benefit studies focused on developing toxin inhibitors.
		2. **Other toxins**Gamma haemolysin and Panton-Valentine leukocidin (PVL) are both two-component toxins that contribute to pathogenesis of *S. aureus* keratitis [(Dajcs et al., 2002; Sueke et al., 2013; Zaidi et al., 2013)](https://paperpile.com/c/Vdx3Lu/szfy%2B5SVM%2BLXss). Zaidi et al. (2013) found that when a topical antibody to PVL was applied to mice 32 hours after infection it reduced corneal pathology for most, but not all, strains when tested at 48 hours. The variability of strain-dependent cytotoxicity was also observed with overexpression of PVL that increased bacterial burden but did not universally affect corneal pathology [(Zaidi et al., 2013)](https://paperpile.com/c/Vdx3Lu/5SVM).

		Gamma haemolysin also contributes to *S. aureus* virulence, whereby the two component proteins (S/F) of the toxin accumulate as pairs adjacent in a cell membrane to form a ring structure that causes lysis [(Sugawara et al., 1997)](https://paperpile.com/c/Vdx3Lu/o3Rj). A contribution to pathogenesis was supported when rabbit corneas infected with a gamma-toxin deficient mutant resulted in a less severe pathology compared to its parent strain [(Dajcs et al., 2002)](https://paperpile.com/c/Vdx3Lu/szfy). The potential for interaction between the bi-component gamma and PVL toxins is known due to their sequence relatedness, but this phenomenon has not been studied in relation to eye disease.

		The contribution of additional *S. aureus* toxins to ocular infection should be investigated. Comparative genomic analysis of *S. aureus* isolates from ocular versus non-ocular infection identified enrichment of multiple enterotoxin genes in the ocular isolates [(Johnson et al., 2021)](https://paperpile.com/c/Vdx3Lu/np15). The isolates included in this study represented the major sequence types: ST5, 8 and 30 showed no association with a particular sequence group. The enterotoxin genes enriched in the ocular isolate group were distributed on enterotoxin gene clusters *egc1* and *egc2* associated with pathogenicity islands [(Johnson et al., 2021)](https://paperpile.com/c/Vdx3Lu/np15). This finding paves the way for genetic and biochemical studies to investigate whether the encoded enterotoxin enzymes are expressed during infection, at what stages, and their overall contribution as potential virulence factors of *S. aureus* ocular disease.
		3. **Proteases**A few studies have investigated roles of staphylococcal proteases in ocular disease. The inhibition of Staphopain A produces a reduction of bacterial numbers and corneal pathology from attachment and invasion of corneal epithelial cells by *S. aureus* [(Hume et al., 2020)](https://paperpile.com/c/Vdx3Lu/IWMl). Separately, SSL1 protein was identified to have serine protease activity and contributed to *S. aureus* corneal disease, possibly due to its effect on host defence and structural proteins [(Tang et al., 2019)](https://paperpile.com/c/Vdx3Lu/tTIw). Serine protease activity associated with the Esp protein of *S. epidermidis* was also proposed to contribute to corneal pathology due to a correlation between the protease activity and severity of damage [(Caballero et al., 2021)](https://paperpile.com/c/Vdx3Lu/tX05).
		4. **Further virulence traits**Greater understanding is needed of the way adhesins contribute to both colonisation and invasion and interaction with ocular mucins. Collagen-binding adhesin contributes to the pathogenesis of *S. aureus* corneal infection [(Rhem et al., 2000)](https://paperpile.com/c/Vdx3Lu/0JAZ), whereas in a comparative genome study a negative correlation was identified between *fnbB*, *sasG* and *sraP*-encoded adhesins and ocular isolates [(Johnson et al., 2021)](https://paperpile.com/c/Vdx3Lu/np15). A combination of genomic and functional studies is awaited to generate further insights of *S. aureus* virulence.
	3. ***Staphylococcus antivirulence targets***
	This understanding of the roles that virulence factors have in pathology has enabled focus on inhibiting function by disrupting their regulated expression by the bacteria. Suvratoxumab (MEDI4893) is an alpha toxin neutralising monoclonal antibody (mAb) that has proceeded to phase 2 clinical trials in patients in intensive care with *S. aureus* pneumonia [(Tkaczyk et al., 2018)](https://paperpile.com/c/Vdx3Lu/VeQt). Other mAb treatments have reached various stages of clinical trials: AR-301 in Phase 3 trials as an adjuvant therapy for *S. aureus* pneumonia [(François et al., 2019)](https://paperpile.com/c/Vdx3Lu/Hsph); ASN-100 mAb cocktail that targets six pore forming toxins, including PVL, LukAB and alpha toxin, has been investigated in clinical trials [(Magyarics et al., 2019; Stulik et al., 2019)](https://paperpile.com/c/Vdx3Lu/IKjp%2BJ1SB). Whether these approaches will have clinical benefit remains to be seen, and substantial further work would be required for their development for ocular infections. Similarly, there have been substantial efforts to generate inhibitors of the Agr regulator system that is key for temporal, density-dependent expression of pore-forming toxins. Approaches to produce analogs of the key signalling auto-inducing peptide and inhibitors of the *S. aureus* AgrC receptor have also been described [(Horswill and Gordon, 2020)](https://paperpile.com/c/Vdx3Lu/zS8c). Small molecules investigated in these studies could have application as adjuvant therapy but remain to be studied for ocular diseases.
	4. ***Potential for identification of virulence in the clinic***Molecular (e.g. PCR) approaches could become a useful tool to identify whether virulence factors are present within the gene pool of clinically infectious agents. In particular, the expression of either ExoS or ExoU genes in a *P. aeruginosa* isolate could guide treatment and/or be an indicator of potential prognoses. *P. aeruginosa* that expresses ExoS can survive inside the host cell, thus escaping antibiotic action and becoming persistent in chronic infections, and strains of *P. aeruginosa* which possess ExoU tend to have higher rates of antimicrobial resistance. Although PCR would be preferable, *P. aeruginosa* in culture can secrete ExoU directly into the culture medium (Schmalzer et al., 2010). This approach could possibly be detected with a phospholipase assay such as with a Cayman chemical cPLA2 assay kit, which would be feasible in most laboratories. A summary of potential anti-virulence therapeutics is presented in Table 3.
3. **CONTROVERSIES SURROUNDING TREATMENT OF BACTERIAL KERATITIS**
	1. ***The purpose of treatment***
	Treatment includes the removal of risk for the infection, killing or disabling the pathogen, minimising associated tissue damage from secondary inflammation, and promoting healing. The course of antimicrobial treatment should also be as short as possible to maximise patient compliance, while minimising the risk of AMR, toxicity, and treatment costs [(A. Lin et al., 2019)](https://paperpile.com/c/Vdx3Lu/LhFq).
	2. ***Selection of treatment***
		1. ***Empiric treatment versus guided treated***
		It is usual to start empiric treatment at presentation before the results of investigations are available. Because of the unreliability of clinical signs, treatment should be effective against the most probable pathogens determined from epidemiological records. This can be guided by clues that determine whether it is likely to be an exogenous or endogenous infection. For example, a keratitis patient who is a contact lens wearer or has been exposed to water, is, apart from risk of acanthamoeba, likely to be associated with a Gram negative bacteria such as *P. aeruginosa* or *enterobacteriaceae,* so that treatment with a fluoroquinolone would be indicated. Use of a single antimicrobial in such a situation that is inactive against Gram negative cover (e.g. chloramphenicol, cefuroxime), may allow rapid disease progression [(Bourkiza et al., 2013)](https://paperpile.com/c/Vdx3Lu/ehoc).

Conversely, a microbial keratitis in a person with ocular surface disease is likely to reflect an endogenous infection from contiguous sites with Gram positive bacteria such as *Streptococcal* or *Staphylococcal spp*. In this scenario antimicrobials such as cephalosporins or glycopeptides would be indicated. This approach, using antimicrobials, which are more selective and effective against the suspected pathogen may be preferable to using broad spectrum antimicrobials or the use of combinations to increase the spectrum of antimicrobial cover. If combinations are to be used, they should be used with the intention of providing a synergistic or additive effect against the likely pathogen, for example, a fluoroquinolone and a carbapenem against *P. aeruginosa*. However, because of the unreliability of clinical signs for decision making, it is unavoidable that a broad-spectrum antimicrobial is used at least until the results of preliminary investigation are available. For broad spectrum cover a meta analysis of RCTs found no evidence of superiority of single to dual therapy [(Hanet et al., 2012; McDonald et al., 2014; Sharma et al., 2016)](https://paperpile.com/c/Vdx3Lu/JsL14%2Bq59p%2BXrHR).

* + 1. ***Choice of topical antimicrobial therapy***
		Sulphonamides were amongst the first antibiotics introduced for topical ophthalmic use, with a marked reduction of the risk of visual loss following traumatic corneal ulceration [(Scott, 1954)](https://paperpile.com/c/Vdx3Lu/PxbE). Penicillin was used subsequently, but this was replaced by cephalosporins in the 1960s due to increasing resistance of *Staphylococcus aureus* to penicillin. Gentamicin was introduced in the 1970s following a rise in *Pseudomonas aeruginosa* infections associated with an increased contact lens wear use [(Baum and Barza, 2000; Erie et al., 1993)](https://paperpile.com/c/Vdx3Lu/hz3p%2BTGNR). Dual therapy was proposed to broaden the range of treatment, with a fortified cephalosporin to treat Gram-positive bacteria and a fortified aminoglycoside to treat Gram-negative bacteria. The evidence for the need to use a fortified concentration of gentamicin (1.4%) is weak and fortified aminoglycosides can rapidly cause ocular surface toxicity, conjunctival necrosis and delayed corneal epithelial healing [(Davison et al., 1991)](https://paperpile.com/c/Vdx3Lu/EvyA).

As monotherapy, the first- and second-generation fluoroquinolones (ciprofloxacin and ofloxacin) were the first to be licensed, with good activity against Gram-negative bacteria and reasonable activity against most Gram-positive bacteria. In addition, and in contrast to gentamicin, they also have good bacteria cell penetration. Subsequently, in the 2000s, the third- and fourth-generation fluoroquinolones (levofloxacin, moxifloxacin and gatifloxacin) were introduced, with a broader spectrum of activity against Gram positive bacteria, but less effective against *P. aeruginosa* [(Baum and Barza, 2000; Kowalski et al., 2003; Mather et al., 2002)](https://paperpile.com/c/Vdx3Lu/hz3p%2BjnmX%2Bwf9f). In terms of MIC, ciprofloxacin currently has the best activity against *P. aeruginosa* for topically licensed antimicrobials (Figure 7) [(Sueke et al., 2010b)](https://paperpile.com/c/Vdx3Lu/UZeN). There are many new antimicrobials which have become licensed for systemic administration and that have particular advantages over currently licensed antimicrobials for topical administration, e.g. the newer generation fluoroquinolones such as delafloxacin, which has a low MIC to both *P. aeruginosa* and *S. aureus*. Use of such antimicrobials off label for topical administration requires the in-house pharmacist to liaise with a medicinal chemist. A list of commonly used licensed and unlicensed (off-label) antimicrobials is provided in Table 1.

Unfortunately, standard laboratory investigation and empiric treatment with a broad-spectrum antimicrobial will not cover all eventualities. Atypical microbial keratitis, defined as rarely isolated organisms, require modified techniques for investigation and treatment [(A. Lin et al., 2019; Ong et al., 2021; Sahay et al., 2020)](https://paperpile.com/c/Vdx3Lu/PX0D%2BLhFq%2Blvvi), and a delay in detection and starting appropriate therapy for *Mycobacterium spp* or *Nocardia spp* may account for the relatively poor outcomes [(Lalitha et al., 2012b)](https://paperpile.com/c/Vdx3Lu/9lPg). Combination therapy for *Mycobacterium spp.* keratitis is usually recommended (e.g. amikacin and ciprofloxacin), although it is not known if these drugs have an additive or synergistic effect or prevent the selection of resistant clones.

Other routes of administration may be required in severe cases (scleral extension, perforation) to achieve levels above the MIC. For example, subconjunctival, sub tenon, intrastromal or intravenous antibiotics have been reported, although these options are not evidence based. Systemic administration (oral or intravenous) is also part of the management of *Neisseria spp*. keratitis or conjunctivitis. Notably, moxifloxacin, a frequently used topical treatment for keratitis, is considered a suboptimal treatment for systemic *Pseudomonas spp.* infections. There have also been alerts regarding serious adverse effects following systemic fluoroquinolones and the risk should be balanced against the uncertain benefit [(Medicines and Healthcare products Regulatory Agency, 2019)](https://paperpile.com/c/Vdx3Lu/1iQc).

* + 1. ***Advantages of combination therapy***
		Although combination therapy, whether simultaneous or sequential, is an option to increase broad spectrum antimicrobial cover, an additional benefit would be to kill the targeted bacteria more efficiently. Selecting a combination that involves two or more different mechanisms, such as interfering with DNA synthesis (fluoroquinolone) and cell wall construction (glycopeptide, beta lactam), has a greater antimicrobial effect and reduces the risk of developing resistance if long-term therapy is required. The result of combination therapy can either be indifferent, additive, synergistic or antagonistic. The nature of the effect is based on the change in the antimicrobial’s MIC when it is used in combination with another antimicrobial. For example, if a combination is indifferent, then there will be no change in the MICs of each antimicrobial when used in combination, but if the combination is synergistic the MIC will be lower. The combination of meropenem (unlicensed for topical use) and ciprofloxacin *in vitro* shows synergy in 20-25% and additivity in 55-60% against *S. aureus* and *P. aeruginosa*. This combination is useful when *P. aeruginosa* shows a high MIC to fluoroquinolones. The combinations of teicoplanin with meropenem, ciprofloxacin, or moxifloxacin also have an additive or synergistic effect of more than 50% against *S. aureus* [(Sueke et al., 2010b)](https://paperpile.com/c/Vdx3Lu/UZeN). However, combination therapy may not always be synergistic or additive, and some combinations are antagonistic and inhibitory and should be avoided [(Kaye et al., 2010)](https://paperpile.com/c/Vdx3Lu/oqb3). For example, an antagonistic combination is fusidic acid and fluoroquinolones [(Ertek et al., 2002)](https://paperpile.com/c/Vdx3Lu/WGnN). The laboratory should report the result of combination sensitivity testing if combinations of antimicrobials are used (Figure 9).

		The increase in AMR has also driven a return to combination therapy. As mentioned previously, in non-ophthalmic isolates there has been a trend toward an increased resistance to the fluoroquinolones, especially for *S. aureus* and *P. aeruginosa,* although the evidence is less clear for ophthalmic isolates [(Garg et al., 1999; Goldstein et al., 1999; Jhanji et al., 2007)](https://paperpile.com/c/Vdx3Lu/4SQc%2BicwY%2B6qMn). As a result the suitability of empiric fluoroquinolone monotherapy has been questioned. In the community MRSA ocular isolates have become more frequent, and *in vitro* the fluoroquinolones are poorly effective against MRSA [(Asbell et al., 2020; Chang et al., 2015; Henry et al., 2012)](https://paperpile.com/c/Vdx3Lu/1jHc%2BI5HR%2B798v). Because MRSA is usually susceptible to glycopeptides such as vancomycin, some centres have reverted to dual therapy, with vancomycin used for Gram positive cover and a fluoroquinolone or aminoglycoside for Gram negative cover [(Saillard et al., 2018; Tam et al., 2017)](https://paperpile.com/c/Vdx3Lu/r3dk%2BPRuV).
	1. ***Other management strategies***
	The first goal of treatment is to achieve medical cure of bacterial infection. However, acute surgical intervention with cyanoacrylate glue or a tectonic keratoplasty may be necessary if there is corneal perforation. Surgical excision of acutely infected tissue has fallen into disfavour [(Cooper and Constable, 1983)](https://paperpile.com/c/Vdx3Lu/mElf). Amniotic membrane transplantation, conjunctival flap or tarsorrhaphy may be used in cases where there is a persistent epithelial defect or ulceration refractory to medical treatment [(Abdulhalim et al., 2015; Gicquel et al., 2007; Kheirkhah et al., 2012; Sheha et al., 2009; Tabatabaei et al., 2017)](https://paperpile.com/c/Vdx3Lu/82aN%2B4Dq2%2BTnkv%2Bt41p%2BdgCL), although it is unlikely they have any role in the elimination of bacterial infection. Other drug delivery options have been suggested to maintain high levels of antimicrobials in the tissue [(Duxfield et al., 2016)](https://paperpile.com/c/Vdx3Lu/E2o5), although the requirement for this in patients compliant with topical therapy is unclear and there are no controlled trials [(Sharma and Taniguchi, 2017)](https://paperpile.com/c/Vdx3Lu/EgPS). These include iontophoresis [(Bertens et al., 2020)](https://paperpile.com/c/Vdx3Lu/HAbJ), nanoparticles [(Ch et al., 2021; Üstündağ-Okur et al., 2015)](https://paperpile.com/c/Vdx3Lu/GfvZ%2BASKk), antimicrobial impregnated soft contact lenses [(Bajgrowicz et al., 2015; Gulsen and Chauhan, 2004)](https://paperpile.com/c/Vdx3Lu/B6jE%2BuhhX) or collagen shields [(O’Brien et al., 1988; Willoughby et al., 2002)](https://paperpile.com/c/Vdx3Lu/m9gE%2BWcLr).
	2. ***Enhanced antimicrobial penetration to a sequestered infection***
	Crystalline keratopathy most frequently occurs when there has been chronic antimicrobial and corticosteroid therapy in the presence of severe ocular surface disease, or after corneal transplantation. The most frequently reported pathogen is *Streptococcus viridans,* with the bacteria in their associated biofilm producing the characteristic appearance. Rarely, diagnosis may require a biopsy for culture and histology. Management includes discontinuation or reduction of the topical immunotherapy and the addition of long-term therapy with a topical antimicrobial [(Porter et al., 2018)](https://paperpile.com/c/Vdx3Lu/DTL0). Intrastromal injection of an antimicrobial, or Nd:YAG laser disruption of the opacities to improve antimicrobial penetration has been suggested [(Masselos et al., 2009)](https://paperpile.com/c/Vdx3Lu/cBlj). An intact epithelial surface may limit antimicrobial penetration to the wound interface following LASIK or SMILE; removing the epithelium or lifting a LASIK flap may enable higher stromal antimicrobial concentrations and is essential to a obtain a sample for culture [(Chandra et al., 2001; Freitas et al., 2003; Ong et al., 2021; Winthrop et al., 2003)](https://paperpile.com/c/Vdx3Lu/TkxB%2BJE1s%2BT2IC%2BPX0D).
	3. ***Antimicrobial prophylaxis for exogenous infection and decolonisation for recurrent endogenous infection***
	Prevention of exogenous infections following injury is based on the use of a broad spectrum topical antimicrobial. In the Bhaktapur Eye Study, patients with a corneal abrasion confirmed by clinical examination and who presented within 48 hours of the injury without signs of corneal infection, were given chloramphenicol ointment 1% three times a day for 3 days [(Upadhyay et al., 2001)](https://paperpile.com/c/Vdx3Lu/5VGY). Eighteen of 442 patients went on to develop corneal ulceration, although the percentage that would have progressed to infection without treatment is not known. The WHO applied the Bhaktapur Eye Study model in Bhutan [(Getshen et al., 2006)](https://paperpile.com/c/Vdx3Lu/CcN9). Volunteer village health workers were trained to to identify corneal abrasions and to use the same chloramphenicol ointment treatment regime. There were 115 corneal abrasions during the 18 month study period in an estimated population of 10,139 and no cases of corneal ulceration in the study population. Based on a previous survey they estimated there would have been 52 cases of corneal ulceration in the study population. This effort is being expanded to other low- and middle-income countries and may be a cost-effective method of preventing the morbidity and further health care costs of bacterial keratitis [(Upadhyay et al., 2007)](https://paperpile.com/c/Vdx3Lu/Db80). Although this supports the use of chloramphenicol as antimicrobial prophylaxis in this population, in countries where Gram negative isolate are common or contact lens wear is prevalent chloramphenicol would be an inadequate prophylaxis and a broad spectrum antimicrobial should be used.

	Colonisation of the nasopharynx, oropharynx, lid margins and ocular surface with pathogenic bacteria, especially *S. aureus*, MRSA, and *P. aeruginosa*, may increase the risk of recurrent endogenous infection [(Kaye et al., 2013; Somerville et al., 2020b)](https://paperpile.com/c/Vdx3Lu/lgT9%2BG6fl). Nasal colonisation with *S. aureus* is much higher in patients with Sjogren’s syndrome, acne, rosacea, psoriasis, and atopic dermatitis with comparative odds ratios compared to control patients of between 1.18 to 14.64 [(Totté et al., 2016)](https://paperpile.com/c/Vdx3Lu/8ShS). It is established that colonisation with *S. aureus* increases the risk of infections particularly endophthalmitis following corneal or cataract surgery. Treatments to decolonize *S. aureus* could be considered in patients with recurrent disease to prevent further infection, although it is unclear whether this lowers the risk of recurrent *S. aureus* bacterial keratitis. Decolonization involves the daily application of a skin disinfectant such as chlorhexidine to the body and hair, and mupirocin three times a day to the nose, both for one week.
	4. ***Risk modification***
	The most important risk factors for corneal infection (trauma, contact lens wear) are potentially modifiable by health and safety and public health initiatives. Endogenous risks such as ocular surface disease are less easy to manage. Wearing protective eyewear for agricultural work would be effective, although unavailable or unaffordable for workers in low-income countries. Withdrawal of inadequate contact lens disinfectant solutions, and education regarding high-risk behaviors (swimming, overnight wear) could substantially reduce the risk of contamination, inflammatory complications and infection [(Arshad et al., 2021; Carnt et al., 2018)](https://paperpile.com/c/Vdx3Lu/NNjM%2Bfuil). The audit of surgical procedures has identified emerging patterns of infection following the introduction of some procedures, such as the risk of *Mycobacterium spp.* infection following LASIK potentially due to contamination of the surgical field with non-sterile tap water [(Nascimento et al., 2018)](https://paperpile.com/c/Vdx3Lu/c5qP). Early referral is also essential, with initiation of effective therapy to minimise tissue damage.
	5. ***Bacterial keratitis in children***
	Managing bacterial keratitis in children can be challenging, and investigation and treatment may require sedation or general anaesthetic [(Al Otaibi et al., 2012; Hong et al., 2012; Yu et al., 2016)](https://paperpile.com/c/Vdx3Lu/INTB%2BTBsC%2BL1Cc). Poor compliance may mean that admission to hospital or subconjunctival injection of antimicrobials are required. There is also the risk of secondary visual deprivation amblyopia [(Hsiao et al., 2007)](https://paperpile.com/c/Vdx3Lu/CLbb). A higher proportion of cases in children are associated with trauma and systemic diseases than in adults, and contact lenses, including cosmetic and orthokeratology lenses, have become significant risk factors in high income countries [(Al Otaibi et al., 2012; Bullimore and Johnson, 2020; Hong et al., 2012; Hsiao et al., 2007; Singh et al., 2020; Yu et al., 2016)](https://paperpile.com/c/Vdx3Lu/CLbb%2BL1Cc%2BTBsC%2BINTB%2BUtUY%2BJFHj), despite the use of antibiotic prophylaxis [(Chen et al., 2021)](https://paperpile.com/c/Vdx3Lu/PzvD). *Pseudomonas* spp. and *Staphylococcus* spp. are common isolates [(Li et al., 2017)](https://paperpile.com/c/Vdx3Lu/Koa3). There is limited data on the prevalence of cosmetic lens or orthokeratology-related keratitis in children although the popularity of contact lenses for myopia-control is increasing [(Van Meter et al., 2008)](https://paperpile.com/c/Vdx3Lu/QUC4). CXL for keratoconus is an emerging risk for *S. aureus* bacterial keratitis in children, especially if there is coexisting atopy and a therapeutic contact lens is inserted for postoperative pain control [(Tzamalis et al., 2019)](https://paperpile.com/c/Vdx3Lu/zD7S). There are no reports of controlled clinical trials to compare antimicrobial therapies in children and we did not identify any published antimicrobial prescription guidelines specific for bacterial keratitis in children (Table 1).
	6. **Antimicrobial stewardship**
	There are a limited range of antimicrobials available for ophthalmic use and resistance to these has increased. Managing the continued increase in AMR through hopeful anticipation of new drug discoveries is not a sustainable strategy. Antimicrobial stewardship refers to the optimal antimicrobial prescription for effective treatment without jeopardising sustainable access to an effective treatment for those who need it [(Dyar et al., 2017)](https://paperpile.com/c/Vdx3Lu/LT06). This also entails reducing unnecessary exposure, minimising the risk of selecting antimicrobial resistance, and conserving some ‘antibiotics of last resort’ (e.g colistin) that are only prescribed when other treatments are likely to fail, rather than the isolate just having a high MIC (see Section 5). Antibiotic stewardship has been shown to limit colonisation by antimicrobial-resistant hospital acquired infections,[(Baur et al., 2017)](https://paperpile.com/c/Vdx3Lu/xanB) although its role in limiting an increase in resistance in ophthalmic isolates acquired in the community remains unproved. In ophthalmology, there is debate regarding the empiric use of vancomycin or teicoplanin as a first line therapy for suspected bacterial keratitis rather than as a therapy for selected cases in which the isolated microorganism has a poor susceptibility to a fluoroquinolone or treatment failure has occurred. Providing the ophthalmologist with the MICs will help address this issue.
	7. **Control of inflammation**
		1. ***Corticosteroid***
		Bacterial infection of the cornea stimulates an inflammatory response that may be exacerbated by bacterial toxins and other virulence factors. For example, exotoxin U produced by *P. aeruginosa* can cause inflammation by releasing arachidonic acid and activation of MAPK signaling, with the production of IL-8 and other cytokines. The inflammation can help eliminate the pathogen but may also contribute to corneal ulceration and scarring. The inflammatory response may be specific to the type of bacteria present and the toxin produced. If there are no viable pathogens then there may be a role for the addition of corticosteroid to reduce inflammation, hasten recovery and preserve vision. Introduction of a steroid in the presence of a viable pathogen may increase the duration of infection and increase the risk reactivation. Wilhelmus summarised the literature from between 1950 to 2000 to help define the role of topical corticosteroid in bacterial infection and presented three key findings: firstly, in eyes with preexisting corneal disease topical corticosteroid increased the odds of ulcerative keratitis (odds ratio [OR], 2.63; 95% confidence limits [CL] 1.41, 4.91); secondly, if corticosteroid had been used before the introduction of an antimicrobial there were increased odds of treatment failure or complications (OR, 3.75; 95% CL, 2.52, 5.58); finally, studies to that date did not show a significant effect of topical corticosteroid on the outcome of bacterial keratitis (OR, 0.62; 95% CL, 0.25, 1.54) [(Wilhelmus, 2002)](https://paperpile.com/c/Vdx3Lu/p0HC). Since then, four randomised clinical trials have addressed the equipoise that exists between the potential gains and associated risks of using topical corticosteroid as part of the treatment of culture-positive bacterial keratitis [(Blair et al., 2011; Carmichael et al., 1990; Srinivasan et al., 2012, 2009)](https://paperpile.com/c/Vdx3Lu/x1C0%2BrfpR%2B8qD3%2BwbEK). All studies used an antimicrobial for 48 hours before steroid was introduced. Three used fluoroquinolone (gatifloxacin or moxifloxacin) hourly for a minimum of 48 hours, with the addition of dexamethasone 0.1% or prednisolone 1% QDS after 48 hours. Patients in the fourth study received a combination of fortified cefazolin (32 g/l) with gentamicin eye drops (14 g/l) hourly, with chloramphenicol at night and subconjunctival cefazolin (125 mg) and gentamicin (20 mg) up to 3 times, and then, if there had been no deterioration after 24 hours, dexamethasone 0.1% QID added. These studies enrolled 612 eyes of 611 patients, with 529 participants reported with a follow-up of 2-12 months. None of the four studies found any important difference between topical corticosteroid or placebo for reduction in ulcer size, time to re-epithelialization, change in visual acuity, adverse events, or quality of life [(Herretes et al., 2014)](https://paperpile.com/c/Vdx3Lu/ASfi).The largest study was the Steroids for Corneal Ulcers Trial (SCUT). This randomised 500 culture proven cases, predominantly from South India, of which 442 completed the study [(Srinivasan et al., 2012)](https://paperpile.com/c/Vdx3Lu/wbEK). However, there was no randomisation according to bacterial species. At 3 months there was no difference in best-spectacle-corrected visual acuity (BSCVA), time to epithelial healing, size of scar or rate of complications such as perforation between the two groups. However, in a post hoc subgroup analysis there was a beneficial effect for eyes with worse vision at enrolment with larger infiltrates in the centre of the cornea. This, however, was not tested for an association with the bacterial species. The study also provides no guidance on the use of topical corticosteroid for culture-negative keratitis. The conclusion was that there is currently inadequate evidence as to support the use of adjunctive topical corticosteroids to improve visual acuity, infiltrate/scar size in eyes with bacterial keratitis. In the absence of evidence, patients who are using a topical corticosteroid when they develop bacterial keratitis should stop the corticosteroid until the susceptibility of pathogen to the antimicrobial has been confirmed and treatment response evaluated, recognising that stopping the corticosteroid will be associated with an increase in inflammation. The following guidelines are still pertinent: 1) avoid a topical corticosteroid if the causative microorganism is unknown or if effective antibacterial therapy cannot be provided; 2) minimize corticosteroid use if the ulcer is away from the visual axis and the ulcer is healing; 3) consider adding a topical corticosteroid if the MIC of the antimicrobial to the isolate is well below the reported corneal concentration of the antimicrobial and if minimisation of visual loss from corneal opacity is considered to be an important enough consideration to outweigh the risks of topical corticosteroid (steroid-induced glaucoma, cataract, infectious crystalline keratopathy, potentiation of unsuspected acanthamoeba, fungal or viral keratitis). In general steroids should be withheld until the clinician is confident that it is unlikely there are any viable pathogenic bacteria, or if re-epithelialization has occurred. For these reasons, antimicrobial/corticosteroid combinations (e.g. dexamethasone/tobramycin, betamethasone/neomycin, dexamethasone/neomycin/polymyxin B) should not be used as empiric first line treatment for suspected MK.
		2. ***Alternatives to topical corticosteroids***Other agents that reduce inflammation without potentiating bacterial growth have been considered. Calcineurin inhibitors (cyclosporin, tacrolimus) reduce inflammation but their effect as an alternative to topical corticosteroid an adjunct to treatment of bacterial keratitis has not been assessed. Non-steroid agents such as oral doxycycline may have a role to counteract corneal stromal melt and thinning by inhibiting matrix metalloproteinases [(Sapadin and Fleischmajer, 2006)](https://paperpile.com/c/Vdx3Lu/fGUR). It is also used to inhibit stromal melt following chemical injury [(Ralph, 2000)](https://paperpile.com/c/Vdx3Lu/tPji), although there is currently no high quality evidence for its use for the management of microbial keratitis [(McElvanney, 2003)](https://paperpile.com/c/Vdx3Lu/kZeg).
1. **ALTERNATIVES AND ADDITIONS TO ANTIMICROBIALS**
With the increase in AMR, attention has turned to alternatives to the current antimicrobials. An alternative should be cheap, stable and non-toxic [(Robaei et al., 2016)](https://paperpile.com/c/Vdx3Lu/PctR). Some alternatives are already available in high income countries with rapid access eye care, as opposed to remote low income areas that have the greatest need. Antivirulence treatments are one such example (see section 6), and others are discussed below.
	1. ***Photoactivated chromophores for corneal cross linkage (PACK-CXL)***
	Corneal collagen cross linkage (CXL) is an effective treatment for progressive keratoconus. The combination of UV light and topically applied riboflavin has a photodynamic effect on the cornea that may also kill microorganisms through the generation of free radicals, and enhance corneal resistance to enzymatic breakdown [(Said et al., 2014; Spoerl et al., 2004; Wollensak et al., 2004)](https://paperpile.com/c/Vdx3Lu/RXnq%2BXmOA%2B4da1). This application of CXL, termed photoactivated chromophores for corneal cross linkage (PACK-CXL), has been proposed as a treatment for corneal infiltrates and early keratitis as an alternative or an addition to an antimicrobial [(Marasini et al., 2021)](https://paperpile.com/c/Vdx3Lu/ZGxv). It may be an additional option for bacterial keratitis refractory to medical therapy, although it will also kill host cells needed for the immune response. As ultraviolet energy (usually 370 nm) is absorbed within the first 100 μm, the effect of cross-linking may be limited to superficial infiltrates [(Papaioannou et al., 2016)](https://paperpile.com/c/Vdx3Lu/YwY5). There are as yet no adequately powered randomised controlled trials to support its use as a primary treatment for suspected microbial keratitis [(Davis et al., 2020)](https://paperpile.com/c/Vdx3Lu/t5hU).
	2. ***Other therapies***
	Defensins are a class of potent antimicrobial peptides, found in the granules of neutrophils and other phagocytic cells, that can kill bacteria by causing membrane damage [(Drayton et al., 2021; Mookherjee et al., 2020)](https://paperpile.com/c/Vdx3Lu/RYif%2BBTIa). Additional antimicrobial peptides have also been found on the surface of corneal epithelial cells and in the tears, where they are thought to act as a component of the innate defence system against bacterial invasion and infection. They are susceptible to protease degradation, and resistance can occur, which may limit their utility. Although promising, there have been no human clinical studies to date of their use in the treatment of microbial keratitis. The option for antimicrobial peptides as an alternative to current antimicrobials has recently been reviewed [(Dijksteel et al., 2021; Fleiszig et al., 2020)](https://paperpile.com/c/Vdx3Lu/fdBK%2ByE9D). Other approaches include the use of novel antimicrobials such as teixobactin [(Fiers et al., 2017)](https://paperpile.com/c/Vdx3Lu/1fuu), or modification of the host response to bacteria with microRNAs [(Drury et al., 2017)](https://paperpile.com/c/Vdx3Lu/k3vw), but how quickly and of these options will move along the research and development pipeline to implementation as a product to treat bacterial corneal infection is unknown.
	3. ***Bacteriophages***
	Bacteriophage or phages are bacterial viruses, and phage therapy has been proposed as an alternative to antibiotics. Independently discovered by Felix d’Herelle and Frederick Twort in the early 20th century [(d’Herelle, 1931)](https://paperpile.com/c/Vdx3Lu/fsz2), they can broadly be classed as temperate (lysogenic) and virulent (lytic) phages. Lysogenic phages can integrate into the bacterial genome and are unsuitable for phage therapy, but virulent phages do not integrate and can cause rapid cell lysis. Phages are less than 200 nm in size and typically consist of a head, tail and tail fibres. They attach to specific receptors on the bacterial cell surface, DNA can then be injected into the bacterium and progeny phage produced. With cell lysis the progeny phages are released. Phage specificity is determined by the receptors it can bind to lipopolysaccharide (LPS) and proteins associated with pili are common targets.
		1. ***Potential Benefits of Phage Therapy***Phages are bactericidal and therefore, unlike some bacteriostatic antibiotics, there is less potential for regrowth. Phage also propagate in areas where the target bacteria are present. The different modes of action to antibiotics mean that phages can be active against multi/extensively drug resistant (MDR/XDR) bacteria. A recent study showed that phage treatment can resensitise AMR pathogens, thereby potentially extending the utility of existing antibiotics [(Gordillo Altamirano et al., 2021)](https://paperpile.com/c/Vdx3Lu/5ALe). The specificity of phages to either a single bacterial species or subset of that species mean that, unlike broad spectrum antibiotics, there is little disturbance of the normal microbiome. However, challenges include the narrow spectrum of phage activity that means they may not be active against all members of a bacterial species, although combinations of phages can be formulated to circumvent this issue and limit the development of bacterial resistance to the phage. This type of therapy could also be applicable to personalised medicine with a bespoke combination of phages. However, these approaches would need to be combined with powerful diagnostics to identify bacteria to the strain level.
		2. ***Barriers to the development and success of Phage Therapy***Phages have an unpredictable ability to evolve during infection. They can mutate rapidly and this trait often allows them to overcome resistance in their bacterial target. However, this has posed a challenge to regulatory bodies as manufacturing of phage preparations to a common standard can be problematic. Concerns over intellectual property may also have led to limited investment by the Pharmaceutical industry. Bacteria can develop resistance and a variety of mechanisms have been described include target modification and CRISPR-Cas systems [(Labrie et al., 2010)](https://paperpile.com/c/Vdx3Lu/8kSR). Not all phages are suitable as a therapeutic agent and detailed characterisation is needed to ensure that a phage cannot integrate into the bacterial genome or carry toxins.

The production of antibodies against phages has been reported. Phage specific IgM and IgG has been detected in several studies and this can reduce the activity of the phage although these effects are not consistent [(Hodyra-Stefaniak et al., 2015; Łusiak-Szelachowska et al., 2017; Żaczek et al., 2016)](https://paperpile.com/c/Vdx3Lu/MPhZ%2B0tRY%2B5Htl). It is unclear what impact this phenomenon might have for surface treatment, such as treatment of eye infection.

* + 1. ***Phage Therapy in the Ophthalmic Setting***There have been a limited number of studies reporting the use of phages for keratitis. KPP12 phage in topical eye drops was investigated in an *in vivo* mouse model of *P. aeruginosa* PA33 keratitis. Treated mice showed a significantly improved clinical outcome including reduced corneal opacity [(Fukuda et al., 2012)](https://paperpile.com/c/Vdx3Lu/feiB). Phage ΦR18 has also been used in an *in vivo* mouse model of *P. aeruginosa* keratitis. Phage administration within 3 hours of bacterial inoculation led to bacterial lysis and the suppression of keratitis, with phages absorbed into the bacterial cells (within 30 secs), and with activity against a wide range of *P. aeruginosa*, including antibiotic resistant isolates [(Furusawa et al., 2016)](https://paperpile.com/c/Vdx3Lu/er7n). In 2015, a case report of a patient with a postoperative corneal abscess infected with *S. aureus* with keratitis, dermatitis and rhinosinusitis over an 11-year period. The patient was treated with *S. aureus* bacteriophage SATA-8505 (ATCC PTA-9476) over 4 weeks and examined at 3 and 6 months. The patient displayed stabilisation of ocular signs and subsequent ocular and nasal cultures were negative [(Fadlallah et al., 2015)](https://paperpile.com/c/Vdx3Lu/0xcb). Phage therapy against *P. aeruginosa* and *S. aureus* has also been investigated in other infection types including respiratory [(Waters et al., 2017)](https://paperpile.com/c/Vdx3Lu/ThQc), wound sepsis, septicaemia and mastitis [(Geng et al., 2020)](https://paperpile.com/c/Vdx3Lu/Xy9G). There have also been some high-profile instances where phages were used as a “last resort” therapy in humans [(Dedrick et al., 2019)](https://paperpile.com/c/Vdx3Lu/QkG7). This has been enabled under *C*ompassionate Use (specified in the Helsinki Declaration of Ethical Principles for Medical Research Involving Human Subjects).
		Phase IV patient safety studies have been conducted [(Petrovic Fabijan et al., 2020)](https://paperpile.com/c/Vdx3Lu/8900), and intranasal phage administration [(Ooi et al., 2019)](https://paperpile.com/c/Vdx3Lu/dFZa), as well as a recent systematic review of phage therapy for superficial bacterial infections suggested that they could be highly effective and that there were no safety concerns when administering purified phage [(Steele et al., 2020)](https://paperpile.com/c/Vdx3Lu/8xoC). Overall, phage therapy provides a promising option as a future therapeutic but key study, particularly for bacterial keratitis, and a clear regulatory pathway are needed before they can be used routinely rather than as an option of last resort.
	1. ***Topical antiseptics and disinfectants***
	In a randomized, controlled clinical trial performed in Philippines and India topical povidone-iodine 1.25% was shown to be a cost effective alternative topical therapy to antibiotics for the treatment of bacterial keratitis [(Isenberg et al., 2017)](https://paperpile.com/c/Vdx3Lu/N2Er). Cationic polybiguanides such as chlorhexidine (0.02% or 0.2%) and polyhexanide (PHMB) are biocides that work by cell membrane disruption. They are inexpensive, easy to formulate and well tolerated [(McDonnell and Russell, 1999)](https://paperpile.com/c/Vdx3Lu/J0UQ). Although primarily evaluated as treatment for fungal or acanthamoeba infection, PHMB has been reported to be effective against *Nocardia* *asteroides* with an MIC of 0.01%. [(Lin et al., 1997)](https://paperpile.com/c/Vdx3Lu/ueP6). Studies of experimental bacterial keratitis treated with chlorhexidine 0.01% have shown that it is as effective as ciprofloxacin or combined cefazolin/tobramycin against *P. aeruginosa* or *S. aureus* [(Bu et al., 2007)](https://paperpile.com/c/Vdx3Lu/OPjJ). Resistance to chlorhexidine has not been systematically evaluated in ophthalmic isolates, although reduced susceptibility in *S. epidermidis* has been identified in isolates from surgical wounds [(Prag et al., 2014)](https://paperpile.com/c/Vdx3Lu/Qrtk). Resistance and allergy to chlorhexidine is reported to be more frequent than to either povidone iodine or PHMB [(Barreto et al., 2020)](https://paperpile.com/c/Vdx3Lu/oi0F), but with more widespread use resistance to antiseptics may increase. Antiseptics are a potential option for low and middle-income countries where a cheap, stable and self-sterilising broad-spectrum antimicrobial may otherwise be unavailable. They may also be useful in preventing infection in patients with compromised ocular surface and in those prone to recurrent keratitis.
1. **DEVELOPING A DIAGNOSTIC RULE FOR BACTERIAL KERATITIS**The interplay of microbial and host factors that determine the health of the ocular surface can make it difficult to distinguish pathogens from contaminants or colonisers [(Chiu and Miller, 2019)](https://paperpile.com/c/Vdx3Lu/PlYi). Understanding this concept is crucial for the interpretation of results from increasingly sensitive molecular diagnostic techniques.
	1. ***The ocular surface microbiome in normal and disease states***Next generation sequencing (NGS) sequencing of the V3–V4 region of the 16S rRNA gene [(Dong et al., 2011; Huang et al., 2016; Ozkan et al., 2017)](https://paperpile.com/c/Vdx3Lu/RMmR%2B2VOk%2BbDez), has established that the ocular microbiome has an unexpectedly diverse microbiome of potential pathogens and commensals that includes *Corynebacterium*, *Acinetobacter*, *Pseudomonas*, *Staphylococcal*, *Propionibacterium* and *Streptococcal spp* [(Delbeke et al., 2021; Dong et al., 2011; Okonkwo et al., 2020; Ozkan et al., 2018)](https://paperpile.com/c/Vdx3Lu/LDEH%2BNRhh%2BRMmR%2B4KI0). Two small shotgun metagenomic sequencing studies of the ocular surface identified *S. epidermidis* in 73%-88% of normal subjects [(Kang et al., 2021; Wen et al., 2017)](https://paperpile.com/c/Vdx3Lu/UweN%2BOap8). Differences in *S. epidermidis* and *S. pyogenes* strains between individuals was also demonstrated [(Kang et al., 2021)](https://paperpile.com/c/Vdx3Lu/UweN). Unsurprisingly, the structure of the microbiome is influenced by age [(Wen et al., 2017)](https://paperpile.com/c/Vdx3Lu/Oap8), gender [(Wen et al., 2017)](https://paperpile.com/c/Vdx3Lu/Oap8), systemic disease [(Li et al., 2019)](https://paperpile.com/c/Vdx3Lu/5Vk1), dry eye disease [(Willis et al., 2020)](https://paperpile.com/c/Vdx3Lu/iqdO), meibomium gland dysfunction [(Dong et al., 2019; Zhao et al., 2020)](https://paperpile.com/c/Vdx3Lu/a2uH%2Bjoh0), contact lens wear [(Shin et al., 2016; Zhang et al., 2017)](https://paperpile.com/c/Vdx3Lu/pKST%2BWrqJ), trachoma infection [(Zhou et al., 2014)](https://paperpile.com/c/Vdx3Lu/qVMp), and the recent use of antimicrobials [(Huang et al., 2016)](https://paperpile.com/c/Vdx3Lu/2VOk). Alterations in the ocular surface microbiome have been demonstrated in patients with traumatic corneal ulcers [(Kang et al., 2020)](https://paperpile.com/c/Vdx3Lu/7abc), fungal keratitis [(Ge et al., 2019; Prashanthi et al., 2019)](https://paperpile.com/c/Vdx3Lu/0197%2BHVUe) and bacterial keratitis [(Ren et al., 2021; Shivaji et al., 2021)](https://paperpile.com/c/Vdx3Lu/Pk97%2BHKLC), although part of the effect may be the result of antimicrobial therapy [(Shivaji et al., 2021)](https://paperpile.com/c/Vdx3Lu/HKLC). The ocular surface microbiome has also been found to differ significantly between contiguous sites, with the eyelid skin having the most abundant bacteria and the conjunctiva having the lowest, but with no difference between limbus and fornix [(Ozkan et al., 2018; Ozkan and Willcox, 2019)](https://paperpile.com/c/Vdx3Lu/L0Xz%2BNRhh).
	2. ***Polymicrobial corneal infection***This can be the culture of multiple bacterial species, or bacteria combined with acanthamoeba or fungi [(Khoo et al., 2020; Lim et al., 2013)](https://paperpile.com/c/Vdx3Lu/PSTj%2BRX9P). Polymicrobial is a term that is normally applied when two or more ‘pathogens’ are isolated from culture, but the results of studies of the microbiome may mean that we have to redefine what is meant by a polymicrobial infection, how we specify the pathogen, or identify which susceptibility result is most relevant when deciding on treatment [(Holmgaard et al., 2020; Li et al., 2018)](https://paperpile.com/c/Vdx3Lu/89oG6%2BJkYH). Although it would be expected that the presence of acanthamoeba or fungi in a polymicrobial infection was associated with a worse prognosis, the effect of a polybacterial culture is uncertain.
	3. ***Lack of concordance between techniques***The results from culture and PCR are often different (discordance rate 27%-66%, see section 3.3.3), either at the point of identifying the presence of a bacterium at all or reporting differences in genus and species, which inevitably complicates management decisions. Where one test is positive and the other negative, ascertainment bias will probably be applied such that more weight is given to the positive result than the negative, but when a different pathogen is identified by culture or PCR both need to be included in the management plan. A better understanding of the corneal microbiome in health and disease may help develop better rules to resolve conflicting results.
	4. ***Subtraction analysis for corneal sample interpretation***When multiple organisms are identified a comparison of microbiological results collected from the affected and unaffected eyes could allow subtractive analysis to be performed to suggest the causative microorganism(s). This would necessitate a sampling technique with minimal risk for the unaffected eye, such as an impression membrane. We have demonstrated this principle in a study where we used corneal impression membranes to take samples from both eyes of 50 patients with unilateral suspected microbial keratitis processed using conventional diagnostic culture. We found *S. aureus*, *S. pneumoniae*, *P. aeruginosa*, *E. coli* and *M. nonliquefaciens* were only cultured from the affected eye, whereas *alpha haemolytic Streptococci*, Diphtheroid and CoNS species were cultured from the unaffected eye in 4%, 4% and 40% of cases respectively (unpublished data). This comparison could be also applied to the more complex NGS analysis, where multiple microorganisms are identified, to indicate the likely pathogenic microbiome in the affected eye. Whether the use of subtraction analysis to identify potentially pathogenic microorganisms could influence treatment decisions needs to be verified and any benefit weighed against the economic cost and the time required to process and compare bilateral corneal samples. In the future, improved understanding of the corneal microbiome of healthy eyes and high-risk groups, such as contact lens wearers, could be used to develop generic data for subtractive analysis without the need for sampling the unaffected eye.
	5. **Artificial intelligence to aid the identification of causative organisms**
	The utility of artificial intelligence (AI), in particular deep learning, has been explored in many of the image-based specialties in medicine, including ophthalmology [(J.-P. O. Li et al., 2021)](https://paperpile.com/c/Vdx3Lu/qDZY). Deep learning has been evaluated for the identification of bacteria with light microscopy and time-lapse images of cultures and colonies to identify potential outbreaks of bacterial infections and monitor antimicrobial resistance profiles [(Egli et al., 2020; Leo et al., 2020; Y. Zhang et al., 2021)](https://paperpile.com/c/Vdx3Lu/fARL%2BrBkI%2BIyPr), with >90% accuracy for diagnosis with Gram stained organisms from blood cultures [(Smith et al., 2018)](https://paperpile.com/c/Vdx3Lu/c7CJ). Machine learning has also been used to process data that link MALDI-TOF profiles to clinical phenotypes, including antibiotic susceptibility [(Weis et al., 2020)](https://paperpile.com/c/Vdx3Lu/fDNh) and defining MICs of organisms including *P. aeruginosa* [(Leo et al., 2020; Smith et al., 2017)](https://paperpile.com/c/Vdx3Lu/VVN1%2BIyPr). Sequencing-based AI has also been shown to significantly reduce the time and computational resources required at various stages of the NGS workflow pipeline [(J. X. Zhang et al., 2021)](https://paperpile.com/c/Vdx3Lu/rtSL); [(Ranjard et al., 2019)](https://paperpile.com/c/Vdx3Lu/fkvQ); [(Guzman and D’Orso, 2017)](https://paperpile.com/c/Vdx3Lu/nRN8); [(Luo et al., 2019; Zomnir et al., 2018)](https://paperpile.com/c/Vdx3Lu/qzbT%2B0xpd), and has been shown to improve prediction of antimicrobial resistance [(Her and Wu, 2018; Kim et al., 2020; Pesesky et al., 2016)](https://paperpile.com/c/Vdx3Lu/W53P%2B6D4W%2B0gRu) and help with the discovery of new antibacterial drugs [(Das et al., 2021; Stokes et al., 2020)](https://paperpile.com/c/Vdx3Lu/fBoW%2BTyTg).

Deep learning can also be applied to the diagnosis of corneal disease. Deep learning has been applied to slit lamp photographs to assist the diagnosis of infectious keratitis and other corneal diseases, with a performance similar to an ophthalmologist [(Gu et al., 2020; Kuo et al., 2020; Z. Li et al., 2021)](https://paperpile.com/c/Vdx3Lu/vK92%2BAYwe%2BJqYv). The diagnosis of fungal keratitis (confirmed by culture) with deep learning has achieved a sensitivity of 70%, which was superior to general ophthalmologists and a similar sensitivity as corneal specialists [(Kuo et al., 2020)](https://paperpile.com/c/Vdx3Lu/vK92). Although these modalities have not yet been validated for real-world deployment, their appeal lies in the simplicity for users, including the use of photographs taken by smartphones, and the relatively limited equipment and infrastructure required. With basic and automated image manipulations, these images can be transferred to a cloud server that contains the deep learning algorithm and there would be only a limited requirement for local data processing. Any network that supports the transfer of images such as 4G or 5G or the internet can be used [(J.-P. O. Li et al., 2021)](https://paperpile.com/c/Vdx3Lu/qDZY). This could provide a valuable resource for regions with limited access to an ophthalmologist although, clearly, this would need to be linked to improved access to appropriate treatments [(Arunga et al., 2019a)](https://paperpile.com/c/Vdx3Lu/qiLw).

Large training and testing datasets are difficult to obtain in bacterial keratitis; an acute-onset disease with poorly defined diagnostic criteria that is not usually subject to systematic image capture. This is not unique to bacterial keratitis. Lack of standardisation and difficulty in image sharing further hinders research efforts, and this is recognised by multiple organisations including the American Academy of Ophthalmology [(Lee et al., 2021)](https://paperpile.com/c/Vdx3Lu/HClL). The Digital Imaging and Communications in Medicine (DICOM) standard is internationally recognised as the standard for medical imaging and has specific agreed criteria for ophthalmic images that should be considered when developing systems with ophthalmic imaging collection. Obstacles to data sharing across institutions and jurisdictions, to grow corneal image datasets, could be overcome if the algorithm trained across many local datasets with only the learning, rather than the raw data, being shared in a process known as federated learning [(Dou et al., 2021)](https://paperpile.com/c/Vdx3Lu/YwDW)**.** Though diagnostic algorithms for bacterial keratitis are still nascent, early consideration should be given to establishing repositories of salient and usable data with standardised formats for images [(Lee et al., 2021)](https://paperpile.com/c/Vdx3Lu/HClL). Machine learning-assisted platforms could also provide better guidance on the appropriate use of antimicrobial therapy [(Feretzakis et al., 2021)](https://paperpile.com/c/Vdx3Lu/thao).

* + 1. **Considerations for algorithm development and deployment**Representative, large and high-quality real-world datasets are fundamental to the development and validation of algorithms that are transferable into clinical practice. Training datasets for diagnosis of corneal infection should include the full range of pathologies and normal anatomy present in anterior segment photographs or other images. Data for validation should be representative of the target population in terms of disease prevalence, racial and gender diversity, and consider technical factors such as image acquisition systems and specifications. A potential bacterial keratitis management pathway integrating digital innovations to assist clinicians in diagnosis and management might include risk factors evaluation via a digitalised questionnaire or chatbot. This could be integrated with slit-lamp images to suggest likely causative organisms, susceptibilities, and potential therapies. Artificial intelligence can further contribute towards diagnostic processes from samples to improve accuracy and/or speed up existing processes particularly where there is limited human expertise, such as reading light microscopy or culture images (see section 3). Proposed therapies could consider local sensitivities using real-time data. Whether AI can be refined to a level to improve the diagnostic pathways and outcomes in scenarios where financial resources are limited remains to be seen.
1. **FUTURE PERSPECTIVES AND CHALLENGES**Bacterial keratitis is a common disease that still results in blinding complications. There is now good evidence that the outcome of bacterial keratitis depends in part on the MIC of the isolated bacteria to the antimicrobial used. Although there are regional differences in the profile of bacteria that are isolated, the relative stability of the spectrum of isolates, the low level of acquired resistance, and the limited range of licenced antimicrobials, means that there are no barriers to the establishment of topical ophthalmic breakpoints. This would lessen reliance on the use of systemic breakpoints to guide the choice of topical antimicrobials, which could potentially lead to improved patient care.However, until ophthalmic breakpoints are available, we recommend that sensitivity testing should report the MIC as well the systemic breakpoint and, importantly, that the MIC should be included in epidemiologic studies to enhance inter-regional comparison. Ideally, continuous surveillance activities for resistance patterns in ophthalmic isolates should be implemented. We also recommend that even though the aetiology of investigation-negative keratitis is unknown these cases should be accurately described and included in epidemiological studies and clinical reports of suspected microbial keratitis. As new technologies are introduced robust comparative evaluation should be conducted in accordance with the Standards for Reporting of Diagnostic Accuracy studies (STARD) [(Bossuyt et al., 2015; Harper and Reeves, 1999)](https://paperpile.com/c/Vdx3Lu/CpW6E%2B1DfxP), using a clearly defined reference standard rather than the untenable concept of a gold standard. The ability of these technologies to influence management decisions and patient outcomes should be integral to these studies. The problem of an increase in AMR will be an inter-generational challenge that extends beyond the field of ophthalmology. Although available antimicrobials may remain the cornerstone of therapy for bacterial keratitis for years or decades, it is conceivable that this will not always be the case. Additional effort, government funding and the use of new technologies, such as deep learning to accelerate new antibiotic discovery, is required to develop alternatives.

Although molecular methods are perceived as the future of microbiological investigation, publications often minimise the significant barriers to their introduction, including their high cost and marginal benefit compared to the current standard of care of culture and sensitivity. Significant cost reductions and advances in informatics will be required before metagenomics is incorporated into the diagnostic pipeline. This is particularly relevant for the low- and middle-income countries that bear the majority of the burden of microbial keratitis. We believe that it is a reasonable supposition that culture and standard sensitivity testing, and the use of topical antimicrobials, will remain the mainstay of management for the foreseeable future. However, advances in molecular technologies, if applied to refine a diagnostic rule, could help determine what microorganisms drive culture negative disease and identify the bacterial specific factors that determine the severity of the infection, with the opportunity to personalise the approach with treatment aimed at specific bacterial virulence factors and toxins. Unfortunately, any increased sensitivity of these investigations could also make it even more difficult to distinguish the true pathogens from a range of potential contaminants from the microbiome. Improvements in non-invasive methods of sampling the cornea could enable a comparison between the healthy fellow and affected eyes for either culture or metagenomics. This would enable subtraction analyses to determine which bacteria are more prevalent and more likely to be pathogenic. Currently, the holy grail of a comprehensive report generated at the point of first contact that informs the clinician or care worker of the likely pathogen(s) and its sensitivity to ophthalmic-appropriate antimicrobials is unattainable.

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**Figure captions**

**Figure 1**

**Determining the breakpoint**

Setting an ophthalmic breakpoint (BP) determines whether the bacteria causing the infection (keratitis) would be regarded as susceptible or resistant to the selected antimicrobial and result in an expected therapeutic success or failure. (A) there is a linear relationship between the minimum inhibitory concentration (MIC) (vertical axis) and the zone of inhibition (horizontal axis). The MIC is inferred from the diameter of the zone of inhibition (ZOI). MR is the MIC used to discriminate between resistant and susceptible bacteria equivalent in this example to a ZOI of 10mm. Bacteria above the MR have smaller ZOI and those below have larger ZOI. (B) If the BP is moved to reflect a higher or lower therapeutic concentration in the cornea, the ZOI corresponding to these BPs will be set at wider and smaller diameters, respectively.

**Figure 2**

**Relationship between the ophthalmic and systemic breakpoints**
Resistant (R) and susceptible (S) *P. aeruginosa* keratitis isolates to a fluoroquinolone. Susceptible means the infection will respond to the antimicrobial at the concentration expected in the tissue. Therefore, this includes the clinical response. The clinical response (healing time according to ulcer size in days/mm2) is on the vertical axis and the log10 of the concentration of the MIC (mg/L) on the horizontal axis. The systemic BP is indicated (long arrow). The bacteria in the grey box (top right) have a MIC above the systemic break point (1mg/L) and also have a poor clinical response (high healing time/ulcer size ratio) and would be regarded as resistant. The bacteria in the red square have an MIC below the systemic break point (1mg/L) and would be expected to be inhibited by the antimicrobial at that concentration. Without considering the ophthalmic clinical response, and because their MICs are below the systemic breakpoint, they would be reported as being susceptible. However, because they have a poor clinical response, they would not therefore be regarded as susceptible. If an ophthalmic breakpoint were to be set at a MIC of 0.18 mg/L (-0.75 as a log10) then they would be regarded as resistant. The bacteria in the green box have tMICs below the systemic BP and also have a good clinical response. If, however, the concentration (and biological activity) of the antimicrobial concentration in the cornea could only reach 0.18 mg/L they would then be regarded as resistant. If an acceptable clinical response time was set at 3 days/mm2, then the bacteria with an MIC greater than this would not be regarded as susceptible. Although the predicted clinical outcome is unknown, if the line of best fit were to be used, then the intersection of the acceptable clinical response time (3 days/mm2) with the slope indicates the MIC that could be used to set an ophthalmic BP [(Kaye et al., 2010)](https://paperpile.com/c/Vdx3Lu/oqb3). The bacteria in the red square have poor outcomes despite having relatively low MICs. Although this could reflect poor antimicrobial activity within the cornea due to poor penetration or reduced activity from protein binding or pH, the high concentration of most topical antimicrobials would make this unlikely. Possible explanations are that it is the result of a poor host response, for example poor healing associated with dry eye disease, or that these bacteria are producing toxins that impair the healing response despite the presence of an effective antimicrobial (Winstanely et al 2005, Stewart et al 2011).

**Figure 3**

**Change in the concentration of an antimicrobial in tissue as a function of time the following administration of a single drop**

1. Shows there is an onset time, which is the interval between drop administration and the antimicrobial achieving an effective tissue concentration. The therapeutic range is the concentration interval between the minimum inhibitory concentration (MIC) and the maximum tolerated concentration (MTC) where toxicity will occur. The duration of action is the time interval when there is a therapeutic concentration in the tissue. The interval between drop applications required to keep the stromal concentration within the therapeutic range is unknown, and this is likely to differ between different antimicrobials and pathogens.
2. Illustrates that the relevant therapeutic drug exposure relates to the time the concentration exceeds the MIC, with total drug exposure expressed as the AUC (MIC), as opposed to the total exposure to any drug expressed as the AUC as seen in A. There may also be a post-antimicrobial effect (PAE). The PAE occurs when the concentration is below the MIC but bacterial growth is inhibited. Therefore, it may not be necessary to maintain the tissue concentration in the therapeutic range, although the effect of the PAE in determining the frequency of drop therapy is also unknown.

**Figure 4**

**The concentration and biological activity of ciprofloxacin in the human cornea and aqueous following topical delivery of 8 doses at 30-minute intervals over 5 hours**

The concentration in the tears 15 min and 30 min after the last drop was 6.7 mg/L, 0.4mg/L. An hour after the last application, the concentration the stroma and aqueous was 14.87 mg/L and 0.51 mg/L respectively, whilst the biological activity (zone of inhibition of growth) was 1.37 mg/L and 1.89 mg/L at the epithelial and endothelial layers. The measured concentration is higher than the measured biological activity [(Kaye et al., 2009)](https://paperpile.com/c/Vdx3Lu/Bcoz), possibly due to protein binding and drug activity at the pH of the tissue.

**Figure 5**

**Corneal stromal and aqueous concentrations following topical delivery of antimicrobials**

The data is a composite from several sources [(Bleeker and Maas, 1955; Holland et al., 2008; Kaye et al., 2009; Kim et al., 2005; Leeming et al., 1994; McGee et al., 2005; Proksch and Ward, 2010; Smith et al., 2001; Stroman et al., 2005; Sugioka et al., 2009)](https://paperpile.com/c/Vdx3Lu/Bcoz%2BRSlh%2BCWfC%2BAPGZ%2BzC7I%2B0kh8%2B5dxF%2BGvPr%2BpRoV%2B1GQe). The number in each column represents the concentration (mg/L). For moxifloxacin, the numbers adjacent to the horizontal lines are the mean and standard deviations from three studies.

**Figure 6**

**The minimum inhibitory concentration (MIC in mg/L) of *S. aureus* keratitis isolates to a fluoroquinolone (ciprofloxacin)**

The concentration of the fluoroquinolone in the aqueous (0.51 mg/L) and its biological activity at the epithelial (1.37 mg/L) and endothelial layers (1.89 mg/L) of cornea. The systemic BP concentration is indicated in the grey bar. Those isolates to the right of the grey bar would be reported as resistant using the systemic breakpoint. Those isolates with MICs above 16 mg/L indicate development of high-level resistance.

**Figure 7**

**An illustration of the importance of defining the antimicrobial when providing the minimum inhibitory concentration (MIC) of the bacterial isolate**

The MIC of *P. aeruginosa* to four fluoroquinolones. Note that for *P. aeruginosa* ciprofloxacin has the lowest MIC. A higher concentration of ofloxacin, levofloxacin or moxifloxacin would be needed in the cornea to inhibit the growth of these *P. aeruginosa* isolates.

**Figure 8**

**The Tube dilution method used to determine the minimum inhibitory concentration (MIC) of a bacterial isolate**

Samples are suspended in broth and the concentration of the antimicrobial in the broth where there is no growth defines the MIC (4mg/L). Tube dilution is still used for many automated instruments.

**Figure 9**

**The arrangement of an agar plate for E-test combination sensitivity testing**

The two E-tests are crossed at 90° at the point of their individual MICs. (A) E-test strip experiments on antimicrobial combinations labeled A and B. FIC of drug A = MIC drug A when tested in combination with drug B divided by the MIC of drug A alone. FIC of drug B = MIC drug B when tested in combination with drug A, divided by MIC of drug B alone FIC = FICA + FICB. (B) synergy: the minimum inhibitory concentration (MIC) of antimicrobial A was 1.0 mg/L when tested alone, but was 0.125 mg/L when tested in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone, but was 0.063 mg/L when tested in combination with antimicrobial A, with a fractional inhibitory concentration (FIC) = 0.25. (C) additivity: The MIC of antimicrobial A was 1.0 mg/L when tested alone, but was 0.5 mg/L when tested in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone, but was 0.063 mg/L when tested in combination with antimicrobial A (FIC = 0.62). (D) indifference: The MIC of antimicrobial A was 1.0 mg/L when tested alone or in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone or in combination with antimicrobial A (FIC = 2). (E) antagonism: The MIC of antimicrobial A was 1.0 mg/L when tested alone, but was 8.0 mg/L when tested in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone, but was 4.0 mg/L when tested in combination with antimicrobial A (FIC = 16). Arrows: the changing MICs.

**Figure 10**

**A genotype map of *P. aeruginosa* isolates**

Keratitis isolates tend to cluster and are more closely related to each other than to isolates from other sites e.g. lung. Each dot represents a clone and two clones connected by a single line differ in only one locus (single-locus variants [SLV]). The locations of keratitis isolates are indicated in red, with the number of keratitis isolates at each location (if more than one) also shown. For example, 2× (arrow) and 5x indicates two and five isolates at one location. The positions of clone D (blue dot) and the reference strains PAO1 (large black dot) and PA14 (red circle with black circumference), indicate that these clone types fall among the keratitis isolates. Most keratitis isolates represent a closely related subgroup possibly from a common ancestor [(Stewart et al., 2011)](https://paperpile.com/c/Vdx3Lu/il3mI). The peripheral ring of dots represents isolates that are mostly unrelated [(Stewart et al., 2011)](https://paperpile.com/c/Vdx3Lu/il3mI).

**Figure 11**

**Infection of a host cell, by *P. aeruginosa* employing the type 3 secretion system (T3SS)**

*P. aeruginosa* makes contact with the target cell, assembles the injectosome and delivers exotoxin virulence factors, which disrupt distinct cellular functions, into the target cell cytosol. In red, agents that could have promise for development as therapeutics which target certain T3SS associated proteins. ExoU (exotoxin U), ExoS (exotoxin S), Rho (rho kinase), Rac1 (member of Rac GTPases), CDC42 (Cell division control protein 42, a GTPase).

**Figure 12**

**Inhibition of exotoxin U of *P. aeruginosa***

Live/dead fluorescence microscopy analysis of scratched human corneal epithelial cells *in vitro* 24 hours following infection, in the presence of varying concentrations of an ExoU inhibitor compound (DMSO, PSA, compound A and B), with moxifloxacin present at the minimum inhibitory concentration (MIC). Dead cells are red and live cells are green. In the presence of DMSO the scratch increases in size with more dead cells despite the presence of moxifloxacin. Note healing of the scratch with an increase in live cells to restore confluence in the presence of increasing inhibitor concentration [(Foulkes et al., 2021)](https://paperpile.com/c/Vdx3Lu/BMPD). This indicates that healing can be promoted by anti-virulence factors in the presence of viable bacteria

**Figure 13**

**Mechanisms of *S. aureus* exotoxin toxicity and potential therapeutics**

*S. aureus* secretes exotoxins that target human cells to induce toxicity and/or facilitate *S. aureus* internalisation during infection. Alpha toxin binds to human ADAM10 receptors in the target cell membrane which leads to Notch activation and proinflammatory signaling. Alpha toxin oligomerises into a ringed hexamer which causes pore formations out of which Na+, K+, Ca2+ and ATP leave the target cell, causing eventual cell death. At higher concentrations of alpha toxin, cell lysis of the target cell can occur. Staphopain A degrades elastin in the cell matrix and results in endocytic uptake and intracellular survival of *S. aureus*. Panton-Valentine leukocidin (PVL) and gamma haemolysin are recruited to the target plasma membrane by fatty acids such as cholesterol. PVL and Gamma haemolysin multimerise to induce pore formations in the target cell plasma membrane, leading to cell lysis.

**Table 1**

**Topical antimicrobials used for the treatment of suspected bacterial keratitis**

This predominantly relates to practice in the United Kingdom, European Union and United States

* In the UK a medicine is either licensed or **NOT** licensed, and all licenced products must have a **Summary of Product Characteristics** (SPC) [(“Products,” n.d.)](https://paperpile.com/c/Vdx3Lu/RjCO).
* UK Licences are either **Product Licences** (**PL** from MRHA) OR **marketing authorisations** (**MA** from EMA).
* The PL or MA covers the **indication**. Very few topical antibiotics are licensed in the UK to treat **keratitis**, hence most of them are used ‘**off label’**
* In the UK – if the indication is for conjunctivitis only, you **cannot** say that the medicine is licensed to treat keratitis
* In the US - a medicine would be “FDA approved”
* An “**FDA approved**” medicine in the UK would be considered **unlicensed** (unless it had a separate UK product licence)
* **Unlicensed** products in the UK could **either** be ‘**compounded to order’** (also known as a ‘**special**’) by a manufacturer who holds the appropriate licence granted by the MHRA **OR imported** via a wholesaler who holds the appropriate licence granted by the MHRA.
* Theoretically any antibiotic product may be ‘compounded to order’ as long as the raw materials and licensed facilities are available, and the law is not broken. The compounded product should be sterile and stable. The formulation is important to optimise effectively and stability.
* Some antimicrobials that are not otherwise licensed may be designated as an **Orphan Drug** for use in specific indications, e.g. polyhexanide for amoebic keratitis [(Anonymous, 2018; Office of the Commissioner, 2021)](https://paperpile.com/c/Vdx3Lu/KU1P%2B87Jk).

**Table 2**

Advantages and disadvantages of current microbe identification methods used for the diagnosis of bacterial keratitis

**Table 3**

Potential anti-virulence therapeutics against major virulence factors of known keratitis pathogens

NAD - nicotinamide adenine dinucleotide, ADP - adenosine diphosphate, GAP - GTPase-activating protein, PLY – pneumolysin, PVL - Panton-Valentine leucocidin