NOVEL APPROACHES IN BACTERIAL KERATITIS

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by

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ואחרון אחרון חביב, תודה להקדוש ברוך הוא, הרופא האמיתי ופוקח עיוורים

DECLARATION

I hereby certify that this thesis constitutes my own work. The material contained in the thesis has not been presented either wholly or in part for the award of any other degree of any institution. The work and ideas of others has been appropriately referenced. Pictures included from patients have appropriate written consent. The research was carried out in the Departments of Eye and Vision Science and Microbiology at the University of Liverpool.

PUBLICATIONS RELATED TO THESIS

Peer reviewed publications

1) Sueke H, Shankar J, Neal T, et al. *lukSF-PV* in *Staphylococcus aureus* keratitis isolates and association with clinical outcome. *Invest Ophthalmol Vis Sci* 2013;54:3410-3416.
2) Kaye R, Kaye A, Sueke H, et al. Recurrent bacterial keratitis. *Invest Ophthalmol Vis Sci* 2013;54:4136-4139.
3) Shankar J, Sueke H, Wiehlmann L, et al. Genotypic analysis of UK keratitis-associated *Pseudomonas aeruginosa* suggests adaptation to environmental water as a key component in the development of eye infections. *FEMS Microbiol Lett* 2012;334:79-86.
4) Kirwan RP, Zheng Y, Tey A, Anijeet D, Sueke H, Kaye SB. Quantifying changes in corneal neovascularization using fluorescein and indocyanine green angiography. *Am J Ophthalmol* 2012;154:850-858.e852.
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6) Kaye S, Gilbert R, Sueke H, Neal T. Orlans HO, Hornby SJ, Bowler IC (2011) *In vitro* antibiotic susceptibility patterns of bacterial keratitis isolates in Oxford, UK: a 10-year review. Eye Jan 21. *Graefes Arch Clin Exp Ophthalmol* 2011;249:797-799.
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Abbreviations

AFLP:	Amplified fragment length polymorphism
API:	Analytical profile index
AT:	Array tube
BSAC:	British Society for Antimicrobial Chemotherapy
BHI:	Brain heart infusion
CA-MRSA:	Community acquired meticillin resistant *S. aureus*
BURST:	Based upon related sequence types
CO_2:	Carbon dioxide
CNS:	Coagulase-negative staphylococci
CoNV:	Corneal neovascularisation
DMEM/F12:	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
ecc:	Extended clonal complexes
ECD:	Endothelial cell density
EDTA:	Ethylenediaminetetraacetic acid
EVA:	Ethylene vinyl acetate
FCS:	Fetal calf serum
FDA:	Food and Drug Administration
FIC:	Fractional inhibitory concentration
HK:	Human keratocytes
HCE:	Human corneal epithelial cells
HPLC:	High performance liquid chromatography
HTS:	High throughput screening
IC:	Impression cytology

ISA: Iso-sensitest agar

LB: Luria-Bertani

MIC: Minimum inhibitory concentration

MLST: Multi-locus sequence type

MGD: Meibomian gland dysfunction

MOG: Microbiology Ophthalmic Group

MRSA: Meticillin resistant *S. aureus*

MSSA: Meticillin sensitive *S. aureus*

MTT: Methyl thiazolyl tetrazolium

ORF: Open reading frame

OSD: Ocular surface disease

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PVA: Polyvinyl alcohol

QS: Quorum sensing

RBC: Red blood cells

rpm: Revolutions per minute

ROI: Regions of interest

RNA: Ribonucleic acid

SD: Standard deviation

SDW: Sterile distilled water

SNP: Single nuclear polymorphism

ST: Sequence type

TBE: Tris borate ethylenediaminetetraacetic acid

TTSS:	Type-three secretion system
UK:	United Kingdom
USA:	United States of America
ZOI:	Zone of inhibition

CHAPTER 1: INTRODUCTION

1.1 Bacterial keratitis

Infection of the cornea caused by replicating bacteria is known as bacterial keratitis. It is a serious ocular condition that can cause significant long term visual morbidity.[1-5]

1.11 Epidemiology

The epidemiological patterns of bacterial keratitis vary with the demographics of the population, socioeconomic factors, geographic location and associated climate. Bacteria are responsible for a larger proportion of corneal ulceration in temperate climates such as the United Kingdom (UK) and the northern United States of America (USA), than in tropical regions such as south India, where fungal infections predominate.[6] An estimated 1500 cases are seen annually in the UK (approximately 250 per year for a city the size of Liverpool or Manchester) and 14,000 in the USA.[7] Complete epidemiological information for developing countries is lacking, however, it is considered to be the leading cause of corneal blindness in developing nations.[8] Whitcher et al,[9] describe bacterial keratitis as the silent epidemic in developing countries.

1.12 Risk factors

Bacterial keratitis rarely occurs in the normal eye because of the cornea's natural and innate resistance to infection (chapter 1.21). Risk factors for keratitis can be categorised into extrinsic factors, corneal disease and systemic disease. Table 1 shows the differences in predisposing risk factors for keratitis in studies from different geographic locations. For example, the proportion of cases of keratitis

associated with contact lens wear was 3% in India compared to 50%, 36%, 22% in France, Switzerland and Australia respectively.

Table 1. Studies that describe risk factors for keratitis. n= number of cases of keratitis

	Bharati[10] (India) n=1043	Bourcier[2] (France) n=300	Schaeffer[5] (Switzerland) n=85	Green[11] (Australia) n=253
contact lens	3%	50%	36%	22%
trauma	28%	15%	20%	16%
corneal disease	67%	21%	29%	18%
corneal surgery	N/A	4%	5%	11%
other causes	2%	N/A	N/A	3%

N/A = risk factor not mentioned in study

1.121 Extrinsic factors

1.1211 Contact lenses

Contact lens wear is the major risk factor for the development of bacterial keratitis in developed countries.[2, 5, 12] Lam et al[12] reported that the incidence of bacterial keratitis was six-fold higher in contact lens wearers than in the general population. There are approximately 36 million contact lens wearers in the USA[13] and 3.7 million in the UK.[14] The annual incidence of bacterial keratitis in contact lens wearers has been reported as being between 10 to 30 per 100,000 in the USA.[15, 16] Contact lens associated keratitis is rarer in developing countries due to a lower frequency of contact lens use.[10, 17, 18] For example Bharati et al,[10] described contact lens wear as only being identified as a cause of keratitis in 3% of a large case series of patients in India, whereas Bourcier et al[2] found contact lens wearers

to constitute 50% of keratitis cases in France. Extended continuous wear soft contact lenses have become increasingly popular, offering increased convenience than daily wear lenses. Extended wear lenses, however, are associated with a higher risk of bacterial keratitis. In the UK, the incidence of keratitis has been estimated at 3.5 per 10,000 in daily disposable users and 20 per 10,000 extended-wear contact lens.[15, 19-21] A case-controlled study by Dart et al[22] showed that the relative risk of keratitis for overnight wear for any lens type, was 5.4 higher than daily use. They also found the risk of vision loss, was lower in the daily disposable group.

Contact lenses invariably cause a degree of corneal hypoxia. This can lead to suppression of epithelial proliferation, reduced corneal integrity,[23, 24] and an increased risk of infection. In addition to contact lens induced corneal changes, some studies suggest that bacterial virulence is enhanced through contact lens use (chapter 4.14). *Pseudomonas aeruginosa* is the most commonly isolated bacterial species in contact lens associated keratitis.[25, 26] It has been shown to adhere to a wide variety of contact lenses[27] and contact lens cases.[28] Choy et al[29] determined that *P. aeruginosa* isolated from patients with keratitis had variations in the type III secretion toxin encoding genes (chapter 4.1222) and Tran et al[30] describe the adhesion to contact lenses by bacterial flagella and pili. Another mechanism which predisposes contact lens wearers to keratitis is by biofilm formation. Bacterial biofilms are structured, surface-associated communities of bacteria which are less susceptible to antimicrobial agents and are protected from the host immune response, giving rise to chronic infections that are difficult to eradicate. The moist conditions in which contact lenses are worn or kept and their storage cases, are ideal for biofilm formation.[31] This allows bacteria to persist on contact

lenses despite the use of disinfecting solution.[32] McLaughlin-Borlace et al[33] evaluated biofilm formation on contact lenses and contact lens cases in 20 patients with bacterial keratitis. 11/20 contact lenses and 17/20 contact lens cases had evidence of bacterial biofilm formation. Biofilm formation occurred with equal frequency with hydrogen peroxide and chlorine release care systems.

1.1212 Trauma

In developing countries however, ocular trauma is the commonest predisposing factor for keratitis.[9] Studies from India and Nepal describe trauma to be causative in keratitis in over 65% of cases.[17, 34] Common causative mechanisms of injury include; corneal abrasions, corneal foreign bodies and corneal lacerations.[35] There is a strong association between agricultural work and the occurrence of bacterial keratitis.[6]

1.1213 Surgery

Corneal surgery is a less common predisposing factor in bacterial keratitis, with reported rates varying between 4%[2] and 11%.[11] Bacterial keratitis after corneal transplantation can severely compromise the survival of a corneal graft (figure 1).[36, 37] Das et al[38] reported that the major risk factors for the development of bacterial keratitis following corneal transplantation are; suture related, failed graft, ocular surface disease and previous Herpes simplex keratitis (HSK).

Bacterial keratitis is a rare complication of laser refractive surgery with an estimated incidence between 1 in 1000 and 1 in 5000.[39, 40] Cataract surgery[41] and corneal cross linking have also been reported as rare causes of cause bacterial keratitis.[42]

1.122 Ocular disease

A variety of ocular diseases can predispose a patient to bacterial keratitis.

1.1221 Ocular surface and lid disease

Ocular surface disease (OSD) is a term used to describe disease resulting from failure of mechanisms responsible for maintaining a healthy ocular surface.[43] It comprises of a range of overlapping conditions that are associated with damage and disruption to the corneal epithelium. OSD can also lead to corneal neovascularisation (CoNV) and predisposes to bacterial keratitis.[2, 10]

Inflammation of the eyelids known as "blepharitis" is commonly present in OSD. The most clinically useful classification of blepharitis subdivides it into anterior and posterior disease. The former is thought to be a product of bacterial overgrowth and/or sebaceous gland activity, whereas the latter is almost always associated with dysfunction of the meibomian glands. Anterior and posterior blepharitis often co-exists. Blepharitis may result in inflammation, altered meibomian gland secretions, dry eye and the development of keratitis.[44]

Abnormalities of eyelid position predispose to keratitis. Inward turning of the eyelid, known as entropion, can be caused by involutional ageing changes or cicatrizing disease such as pemphigoid. The resultant misdirected eyelashes may abrade the cornea, leading to epithelial breakdown and subsequent infection. Other lid position abnormalities that can cause ocular surface problems are ectropion (outward turning of the eyelid) and lagophthalmos (inability to close the eyelid). All of these predispose to the development of bacterial keratitis.

1.1222 Corneal disease

A neurotrophic cornea is a significant risk factor that may predispose to bacterial keratitis.[45] Common causes of a neurotrophic cornea include previous viral infection (e.g. HSK), topical anaesthetic abuse and trigeminal nerve palsy. Similarly, corneal dystrophies such as lattice and epithelial basement membrane dystrophy, can result in a recurrent corneal erosion and a poor ocular surface. This may predispose the cornea to bacterial infections.[46]

Conditions that damage the corneal limbal stem cells can result in epithelial defects and subsequent corneal ulceration.[47] Hereditary causes of limbal stem cell disease include; aniridia, ectodermal dysplasia and keratitis-ichthyosis-deafness syndrome.[48] Acquired causes of limbal stem cell deficiency are more common and include chemical and thermal burns, multiple ocular surgeries involving the limbal region, contact lens wear and ocular surface inflammatory diseases. Systemic inflammatory diseases that cause limbal stem cell failure include Stevens-Johnson syndrome[49] and mucous membrane pemphigoid.[50,47, 51]

1.123 Systemic disease

A wide range of systemic diseases are thought to predispose patients to bacterial keratitis.[2] Diabetes mellitus was shown in various studies to predispose to bacterial keratitis.[52, 53] Diabetes mellitus can lead to OSD through a reduction in the quality and quantity of the tear film, conjunctival squamous metaplasia and goblet cell loss, and neurotrophic keratopathy.[53] Bourcier et al[2] found that diabetic patients presenting with keratitis were significantly more likely to have “very poor” visual outcomes, compared to non-diabetic patients.

Connective tissue disorders such as rheumatoid arthritis, systemic lupus erythematosus, polyarteritis nodosa and granulomatosis with polyangiitis (formally called Wegener's disease[54]) have all been implicated in bacterial keratitis.[55, 56] The exact pathogenesis of keratitis in these disorders is not yet clear, but circulating immune complexes are thought to result in peripheral corneal melting and a predisposition to severe corneal inflammation and infection. Other systemic diseases predisposing to bacterial keratitis include disorders such as rosacea and atopy. Systemic causes of limbal stem cell failure have been previously discussed.

1.13 Clinical features

1.131 History

Obtaining a detailed history is essential in evaluating patients with clinically suspected bacterial keratitis. Pertinent information that should be elicited include; ocular symptoms such as pain, redness, discharge, blurred vision, photophobia, duration of symptoms and circumstances surrounding the onset of symptoms. If contact lenses have been worn, a detailed history is essential, in particular the type of contact lens worn, wearing schedule and contact lens hygiene. A review of the ocular history may point to underlying aetiology of keratitis, for example, previous viral or bacterial keratitis, trauma, ocular surface disease and previous ocular surgery. The use of current and recently used ocular medications such as topical steroids, antimicrobials and anaesthetics is pertinent. A review of general medical history should be obtained including immune status, and the presence of underlying inflammatory conditions such as rheumatoid arthritis.

1.132 Physical examination

Physical examination of a patient with suspected bacterial keratitis should include measurement of visual acuity, an external examination, and slit-lamp biomicroscopy. Although visual acuity is often compromised due to tearing, discharge, photophobia and patient discomfort, it is essential that it is documented. External examination should be performed, starting with the general appearance of the patient. Skin conditions such as acne rosacea may suggest *Staphylococcus aureus* infection.

Slit-lamp biomicroscopy should be proceed in a systematic “front to back” manner. The lid margin should be examined for the presence of meibomian gland dysfunction and blepharitis. Conjunctiva should be examined noting the extent and position of any inflammation, discharge, follicles, papillae, foreign bodies, and evidence of previous surgery such as blebs or glaucoma drainage devices. Scleral inflammation and thinning may be associated with connective tissue disorders. Corneal sensation should be tested, the absence of which is a significant risk factor and may be suggestive of neurotrophic diseases such as previous HSK. The corneal epithelium should be examined before and after the application of fluorescein or other vital dyes (figure 1). The presence and extent of stromal infiltration and thinning should be assessed. Severe keratitis may result in corneal thinning, descemetocele and even frank corneal perforation. Signs of previous surgery (including refractive), foreign bodies, sutures and underlying corneal dystrophies should also be looked for. The anterior chamber should be assessed for depth and the presence of inflammation including cell, flare, fibrin and hypopyon. The anterior vitreous should be examined for the presence of inflammation. The contralateral eye should be examined in a similar manner,

which may uncover asymptomatic disease and clues to the aetiology. Chapters 3 and 4 describe the characteristic features of *S. aureus* and *P. aeruginosa* associated keratitis.

1.133 Documentation

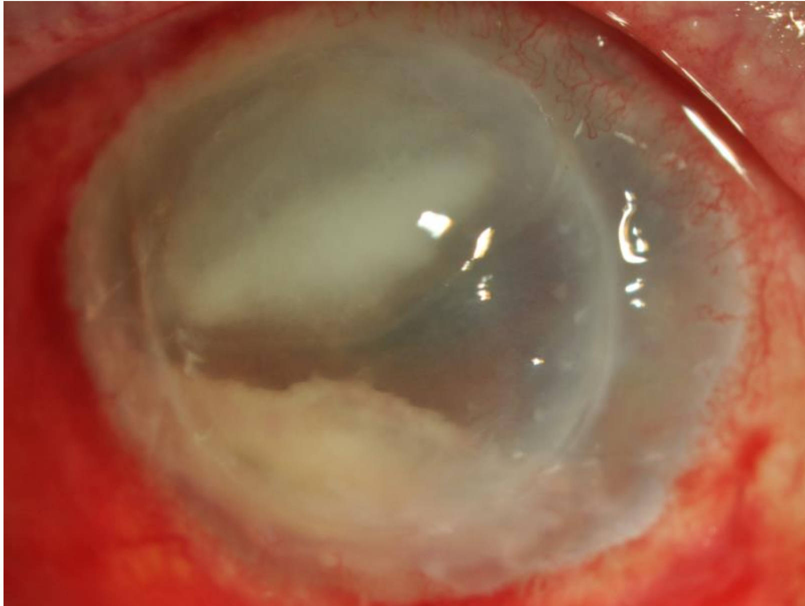
Accurate documentation of the clinical appearance of bacterial keratitis is essential in order to monitor the patient's response to treatment. Management decisions are often made on the basis of documented changes in the examination findings. Furthermore accurate documentation is invaluable when investigating new therapies or virulence factors in the disease (chapters 3 and 4). Documenting the clinical characteristics of a patient with pathological corneal disease was standardised by a colour coding system described in 1977 by Warring et al[57] (figure 2). Using this system allows particular features to be documented in patients with bacterial keratitis; measurement of epithelial defect (mm), distance from the limbus (mm), stromal infiltration, corneal oedema, anterior chamber activity, CoNV and corneal thinning.

1.134 Differential diagnosis

Pathogens other than bacteria need to be considered as causative agents in keratitis. Specific clinical features may point to other aetiologies, for example: reduced corneal sensation in HSK and perineural infiltrates in acanthamoeba keratitis. Ring infiltrates are most consistently associated with acanthamoeba keratitis, but they have also been described in fungi, HSK, varicella zoster, immune related conditions such as rheumatoid arthritis and Gram negative bacilli infection such as *P. aeruginosa* and *Moraxella.*[58-60] In more than half of patients with a clinical diagnosis of keratitis a causative microorganism is not isolated.[61]

This may be either due to limitations in diagnostic techniques, or due to the ulcer being truly “sterile”. In the absence of a positive culture it may be difficult to differentiate the two.

(a)



(b)

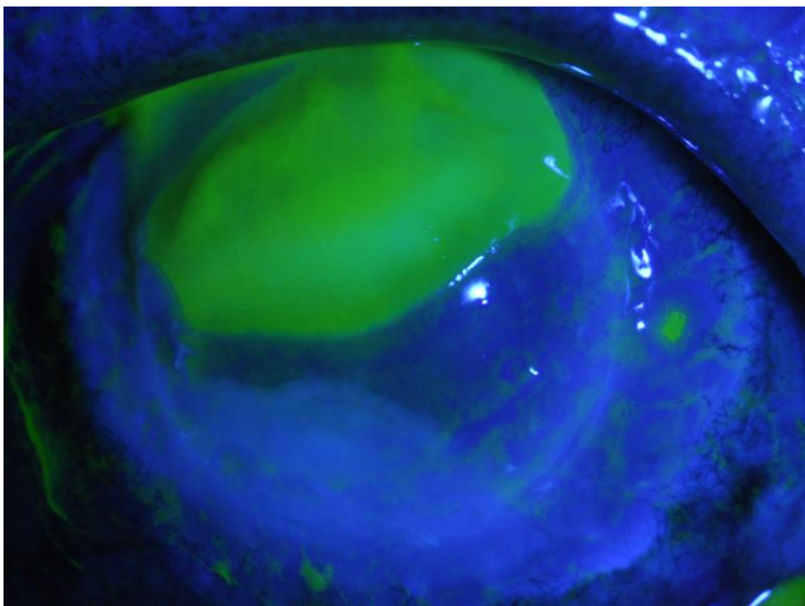


Figure 1. (a) photograph of severe bacterial keratitis in a patient with a previous corneal transplant due to *P. aeruginosa.* Clinical features; dense conjunctival injection, dense stromal infiltration and abscess, hypopyon, corneal neovascularisation and meibomian gland disease. (b) photograph under cobalt blue light illumination showing a large epithelial defect highlighted by the application of fluorescein.

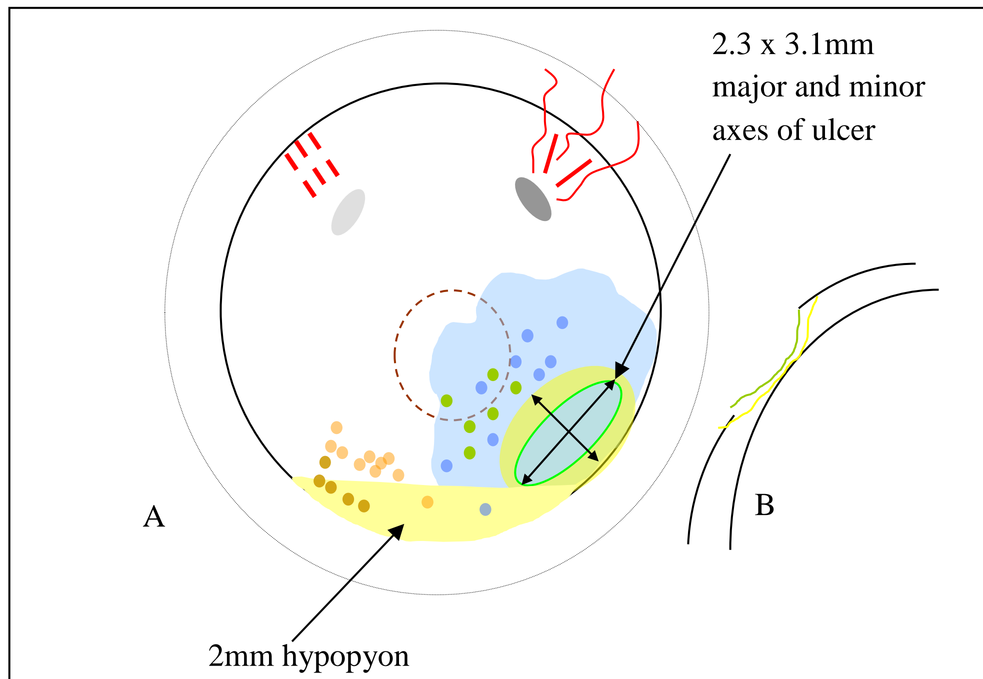


Figure 2. Schematic of a corneal ulcer adapted from Warring et al.[57] A: anterior posterior view, B: cross section through ulcer. Black continuous circle, corneal limbus; outer dashed line, contact lens. Blue shade, stromal oedema; blue dots, epithelial oedema. Green dots, punctate keratopathy; green line, epithelial defect. Red straight hashed lines, ghost vessels; straight lines, deep stromal vessels; wavy lines, superficial vessels. Grey oval shapes: light grey, old scar; dark grey recent scar. Orange and brown dots: new and old keratic precipitates. Yellow shade: hypopyon, corneal infiltrates and abscess formation. Brown hashed circle: pupil.

1.14 Investigations

1.141 Corneal scrape

Sampling of the ulcer in a patient with keratitis is necessary to identify the causative microorganism and plan treatment. This is typically done by "corneal scraping", which is described in chapter 2.11. In addition to its diagnostic value, scraping may enhance antimicrobial penetration and the therapeutic debridement

of infected tissue. The need to detect bacterial, fungal, and amoebic pathogens, coupled to the fact that there may be only a few organisms in a corneal ulcer, means that adequate clinical material must be obtained and cultures must be grown on a variety of media. This has led to the traditional practice of taking multiple scrapes from the ulcer and directly plating onto several culture media. Allen and Dart,[62] for example, suggest that scrapes should be put on a glass slide for Gram staining, an agar plate for aerobic incubation and other culture media to identify less common pathogens.

Collecting multiple scrapes from the eye of an uncooperative patient is not always easy and growing samples in culture on an agar plate is technically difficult. In addition, a full range of fresh culture media is not always instantly to hand. Kaye et al[61] investigated a method of indirect inoculation of brain heart infusion (BHI) liquid media followed by plating on solid media, to recover bacteria. BHI broth has the advantage of a longer shelf life than solid media. Kaye et al[61] found that there were no significant differences in the number of positive cultures of the indirect inoculation BHI method compared to direct inoculation of bacteria onto solid media. They suggested that where there is difficulty to collect multiple scrapes with plating onto several media, the use of BHI with subsequent laboratory plating followed by an enrichment broth for organisms that are difficult to culture, may be sufficient to isolate the causative microbe.

Some authors have investigated using polymerase chain reaction (PCR) techniques in order to improve the sensitivity of diagnosing and characterising keratitis.[63, 64] Kim et al[64] showed that out of 108 samples taken from patients with bacterial or fungal keratitis, 25 were culture-positive for bacteria and 37 were

positive for PCR. The majority of sequenced PCR products matched the positive culture results.

Microbiological methods of identifying bacteria isolated from patients with keratitis are discussed in chapter 2.12. Chapter 6.412 discusses novel molecular diagnostic techniques that can be used to identify bacteria.

1.142 Corneal biopsy

In severe progressive keratitis, where corneal scrape techniques have failed to identify causative microorganisms, a corneal biopsy is indicated. Allen and Dart[62] suggest that biopsies should be excisional (remove in total) in the case of peripheral lesions, and incisional (remove in part) in the case of larger lesions involving the visual axis. Bacteria, fungi, and protozoa can all be visualised in biopsy specimens using light microscopy. Histological analysis is normally available within 48 hours, whereas culture of fastidious organisms may take several weeks. An important secondary role of a corneal biopsy is the debridement of necrotic tissue, which in itself can be a significant aid to healing. Corneal biopsy may lead to irregular astigmatism, although this needs to be weighed against potentially much greater complications in untreated keratitis.

1.143 Corneal imaging

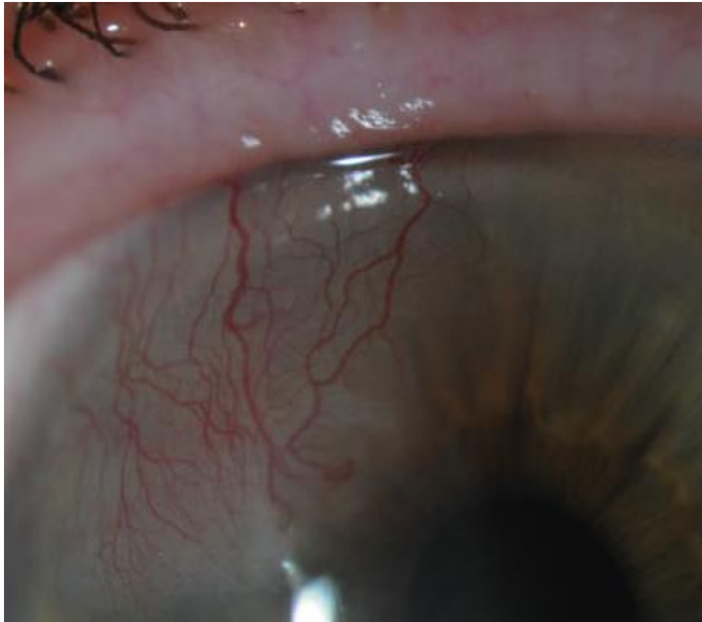
1.1431 Scanning confocal microscopy

Scanning laser confocal microscopy is used to image the various levels of the cornea from epithelium through the stroma to the endothelium *in vivo*. Recent advances have enhanced the resolution and microscopic power of the confocal microscope, which has extended its use as a diagnostic tool in keratitis, particularly to detect the presence of fungi and acanthamoeba.[65, 66]

1.1432 Angiography

CoNV is commonly associated with exudation and the development of corneal scarring during and following bacterial keratitis. It has been estimated that in the United States, 1.4 million patients per year develop CoNV; 12% of them also experience a decrease in visual acuity.[67] A variety of methods have been proposed to reduce their formation or to enhance regression for example, topical steroids,[68] photodynamic therapy,[69] and vascular endothelial growth factor inhibition.[70] Proper documentation of CoNV is therefore important. CoNV associated with keratitis is is commonly analysed by observing photographic images of the cornea. This technique, however, is relatively insensitive and many of the blood vessels, particuarly the thin afferent vessels are not visible. In addition, the presence of corneal scars limits the the detection of CoNV on photographic images. The use of corneal angiography, using fluorescein and indocyanine green dyes, has recently been shown to be a valuable tool in determing the extent of CoNV and its response to treatment.[71] [72] Figure 3a shows a picture of a cornea with CoNV. Fluorescein angiography of the same cornea shows more extensive vascularisation that was observed on the colour picture. Angiography is also useful in deliniating the major feeder or afferent vessels in a patients with CoNV and in assessing vessel maturity and activity.[13,14] These afferent vessels may then be ameanable to treatments such as fine needle diathermy, which may help reduce the leakage and exudation of lipid and other substances into the corneal stroma.

(a)



(b)

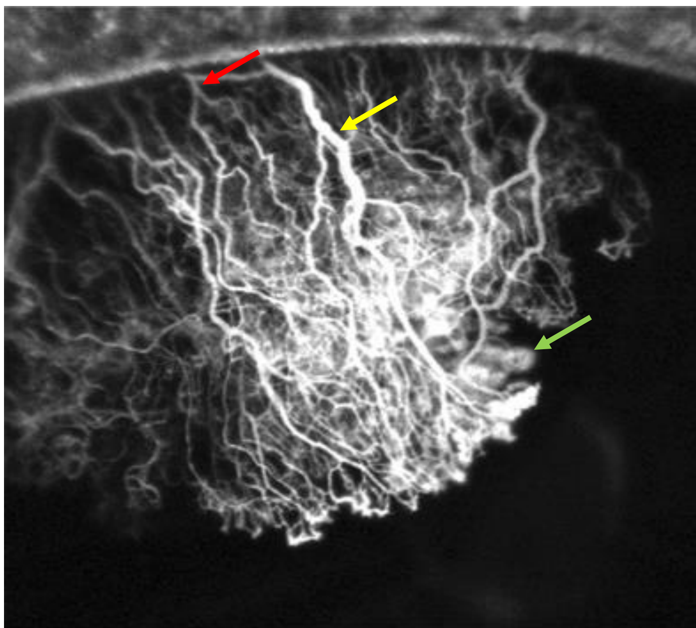


Figure 3: (a) Colour photographs of corneal neovascularisation (CoNV) following bacterial keratitis. (b) Corneal fluorescein angiography showing extent of CoNV. Example of an afferent vessel indicated by a red arrow. The larger vessels (yellow arrow) are efferent draining vessels and are more numerous. Note apical leakage (green arrow) from immature blood vessels.

1.15 Management

The management of bacterial keratitis is multifaceted. It includes preventative measures, medical treatment (mainly in the form of topical antimicrobials) and surgical intervention in severe cases. An excellent approach to management of microbial keratitis has been described by Allen and Dart[62] which will be discussed below.

1.151 Prevention

Avoiding or treating predisposing factors may reduce the risk of bacterial keratitis. Dart et al[22] found 30% of 813 daily disposable users to be incorrectly using their lenses for overnight wear. Overnight wear of daily disposable lenses is an off-label use that increases the risk of developing bacterial keratitis. This emphasises the importance of educating patients regarding the correct use of contact lenses,[7, 73,74] which may in turn reduce their incidence of bacterial keratitis.

Early detection and appropriate treatment of bacterial keratitis are important to minimize permanent visual loss.[75] OSD, blepharitis, lid position abnormalities and predisposing systemic diseases should be all be treated to reduce the risk of bacterial keratitis. Patients with predisposing risk factors should be educated about their increased risk, be acquainted with symptoms of infection, and be informed that they should consult an ophthalmologist promptly if they experience these symptoms.[76]

1.151 Medical treatment

Frequent application of a topically applied antimicrobial, is the mainstay of treatment for bacterial keratitis. The initial antimicrobial used is selected based on the most likely causative bacteria from contemporaneous clinical and laboratory

data (bacterial spectrum and antimicrobial studies) and knowledge of the pharmacokinetics and pharmacodynamics of the agent. Treatment is then modified based on the actual bacterium identified, antimicrobial susceptibility assay data and clinical response. Combination therapy, where two antimicrobials are simultaneously used in a patient, is sometimes used. The advantages of combination therapy over monotherapy are; (1) increased bacterial coverage and (2) the possibility antimicrobial synergy (chapter 5.126). Antimicrobials used in keratitis are discussed in chapter 5.

Allen and Dart[62] suggest that the initial therapy in bacterial keratitis may be divided into two distinct phases:

Phase 1, known as the sterilisation phase, is a period of intensive antimicrobial treatment designed to sterilise the cornea. Antimicrobial therapy should be given hourly for 5 days followed by 4 times a day until epithelium healed. In severe cases, topical antimicrobials should continue through the night and systemic antimicrobials should be given. Patients should be reviewed at 48 hours and 1 week, except for those with rapidly progressing disease who should be seen daily.

Phase 2, known as the healing phase, aims at limiting further inflammatory damage, preventing re-infection, and promoting epithelial healing. During this phase, Allen and Dart[62] recommend reducing antimicrobial treatment to prophylactic levels, in order to reduce corneal toxicity of drops. They also suggest using unpreserved medication and treating predisposing features such as OSD. The cautious use of topical steroids may also be considered at this stage. Srinivasan et al[77] randomised 500 patients with bacterial keratitis in India, to receive either placebo or topical steroid. They all had previously been treated with intensive topical moxifloxacin for 48 hours. They found that there was no overall

difference in 3-month best corrected visual acuity and no safety concerns with adjunctive topical steroid therapy for use in bacterial keratitis. It is important to note that steroids may increase the pathogenicity of keratitis caused by acanthamoeba.[78] The treating clinician must ensure the absence of these pathogens prior to commencing topical steroid treatment.

1.152 Surgical treatment

Patients with severe bacterial keratitis, especially those at risk of corneal perforation, may require surgical intervention. Impending or small perforations can be sealed by cyanoacrylate tissue adhesive. As well as providing tectonic support, cyanoacrylate has been shown to have bacteriostatic properties which may be beneficial in a patient with bacterial keratitis.[79] Amniotic membrane may also be used to cover an impending or small perforation.[80]

In patients with large corneal perforations corneal transplantation is occasionally needed. Bacterial keratitis accounts for approximately 8% of corneal transplants undertaken in the UK [Ocular Tissue Advisory Group to NHS-BT, UK] and 1% in the USA [Eye Bank Association of America]. An emergency penetrating keratoplasty performed at the acute stage of bacterial keratitis is associated with a higher complication rate and lower graft survival. If it is possible, penetrating keratoplasty is deferred until the acute infection has responded to treatment.

1.2 Structure and function of the ocular surface

The cornea is a specialised transparent tissue situated at the front of the eye (figure 4). It provides structural integrity to the globe, protects the inner components of the eye from injury and focuses light onto the retina. The cornea

covers the anterior 15% of the surface area of the eye and is continuous posteriorly with the white opaque sclera. When viewed anteriorly the cornea appears elliptical, its largest diameter is typically in the horizontal meridian (approximately 11 mm) and its smallest is in the vertical meridian (approximately 10 mm). The cornea has an average thickness of 550 µm and is arranged into five layers, distinct in both structure and function; epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. The cornea is composed of three major cell types: epithelium, keratocyte and endothelium which vary considerably in their capacity to regenerate. Epithelial cells have the highest rates of cell division and endothelial cells are the least renewable.

The external surface of the cornea is covered by a 4-6 cell layered stratified squamous epithelium. Epithelial cells are formed from limbal stem cells and upon maturation they migrate from the limbal region towards the centre of the cornea and the apical surface. The corneal epithelium is a smooth, non-keratinized 50 µm thick layer, allowing it to function as a transparent, avascular surface. Basal epithelial cells actively secrete a basement membrane, composed of type IV collagen fibrils, laminin, heparin sulfate, and fibronectin. The cytoplasm of all epithelial cells contains mainly cytoskeletal intermediate filaments and has sparse cytoplasmic organelles, which aid in maintaining transparency. The epithelial cells are adherent to each other by desmosomes, while the basal surface of the epithelium adheres to the basal lamina and underlying Bowman's layer through hemidesmosomes. Zonula occludens tight junctions are also present between epithelial cells in both apical and basal cells.[81] They are made up of the tight junction proteins occludin, and claudin which eradicate the intercellular space. These tight junctions provide an almost impenetrable barrier for invading

pathogens, which explains why the healthy cornea is resistant to infection despite it being in a constant state of regeneration. The corneal epithelium is also the most important layer when considering the penetration of topically applied drugs into the eye. This is discussed in chapter 5.

The stroma accounts for 90% of the corneal thickness. It is predominantly composed of water and contains a structured network of collagen, keratocytes, proteoglycans, corneal nerves and glycoproteins. Corneal transparency is attributed to the regular lattice-like arrangement of collagen fibrils in the stroma. Keratocytes are interconnected by cellular processes divided by gap junctions that only allow very small molecules to penetrate to its neighbouring cells. Keratocytes may secrete matrix metalloproteinase when activated by microorganisms, which coupled with a damaging immune response may cause the degradation of the corneal stroma.[82]

The cornea is bathed in the tear film and comprises an outer lipid layer produced by the meibomian glands, a middle aqueous layer produced by the lacrimal gland and an inner mucin layer produced by conjunctival goblet cells. The tear film plays a critical function in the health of the cornea, supplying it with oxygen and nutrition as well as protecting it from invading pathogens. Indeed the tear film possesses many nutrients that could potentially foster bacterial growth, however, the corneal surface, unlike the skin, contains relatively few bacteria. Mucins, defencins, immunoglobulins, complement and surfactants present in the tear film create an environment that is inhospitable for bacteria to survive.[83, 84, 85] Moreau et al[86] postulated that the tear film enzyme phospholipase A_2, may be the most important barrier protecting the eye from bacteria. They found that the amount of phospholipase A_2 increases in the tear film in a rabbit model of

keratitis, thus enhancing protection against bacteria. The protective properties of the tear film against bacteria is highlighted by the observation that certain bacteria can easily invade multi-layered corneal epithelial cells grown *in vitro* in the absence of the tear film.[87, 88]

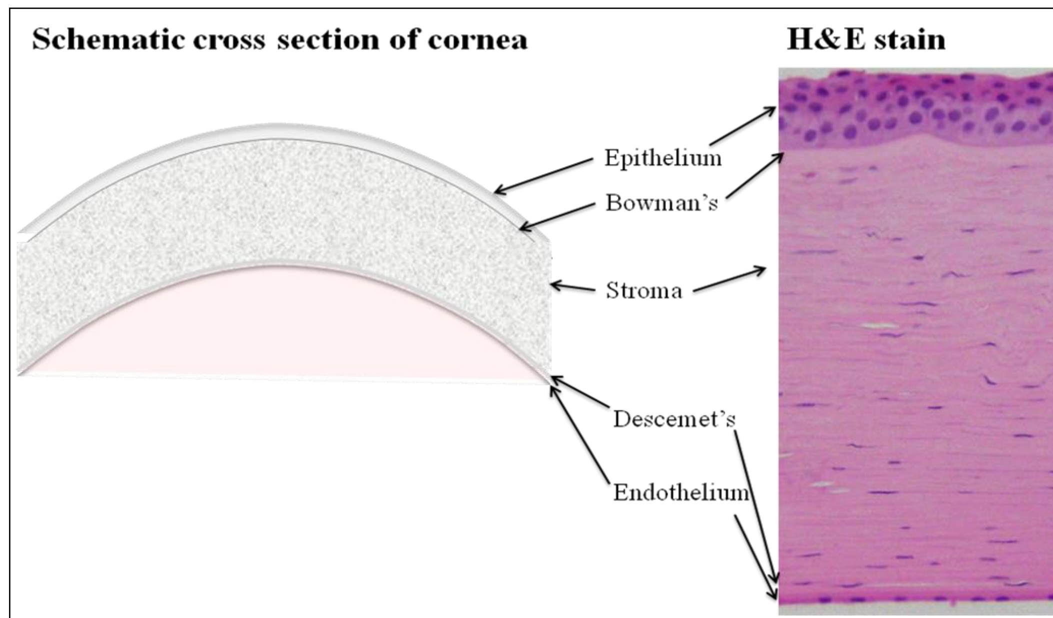


Figure 4: schematic cross section of a human cornea and histological section of the cornea with hematoxylin and eosin stain.

1.3 Bacteria

1.31 What are bacteria?

Bacteria are unicellular microorganisms measuring a few micrometres in length that belong to the kingdom Protista. Bacteria are extremely successful colonisers being present in even the most inhospitable conditions such as the deep ocean and hot springs.[89] Many bacteria live in symbiosis with a host, some however, are parasitic.

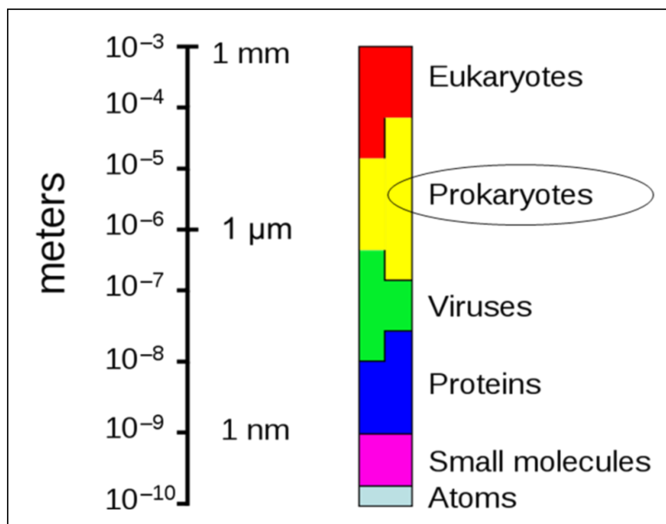


Figure 5. Approximate size of prokaryotes in relation to other cells and particles

Bacteria do not contain mitochondria, endoplasmic reticulum, golgi bodies, nuclear membrane or a true nucleus and are therefore considered to be prokaryotic (Figure 5). The bacterial cell wall is essential in providing protection to the organelles against the host's immune system and antimicrobials. Two major cell wall types exist which subdivide bacteria into two main groups: Gram-negative and Gram-positive. Figure 6 illustrates the main differences between the two groups. Gram-positive bacteria have a cell wall that contains a thick peptidoglycan layer (50-90% of the cell envelope). Their walls also contain teichoic and lipoteichoic acids, which are also unique to these bacteria. Gram-negative bacteria, however, have a thinner peptidoglycan layer (10% of the cell envelope). Their walls also possess a phospholipid outer membrane, which forms an extra protective barrier conferring resistance to enzymes, toxic substances and some antimicrobials. The periplasm, a layer between the inner and outer walls of the cell membrane, is also unique to Gram-negative bacteria, containing enzymes that cause the inactivation of some antimicrobials. The outer membrane of Gram-

negative bacteria contains the lipopolysaccharide endotoxin, which contributes to bacterial virulence.

Pili (also known as fimbriae) are hair-like appendages found on the surface of many Gram positive and Gram negative bacteria.[90] They are long filamentous structures containing oligomeric pilin proteins. Flagella are whip like extensions that act as locomotive organelles, allowing bacteria to move toward favourable environments. Pili[90] and flagella[91] have been implicated in bacterial virulence (chapter 1.333).

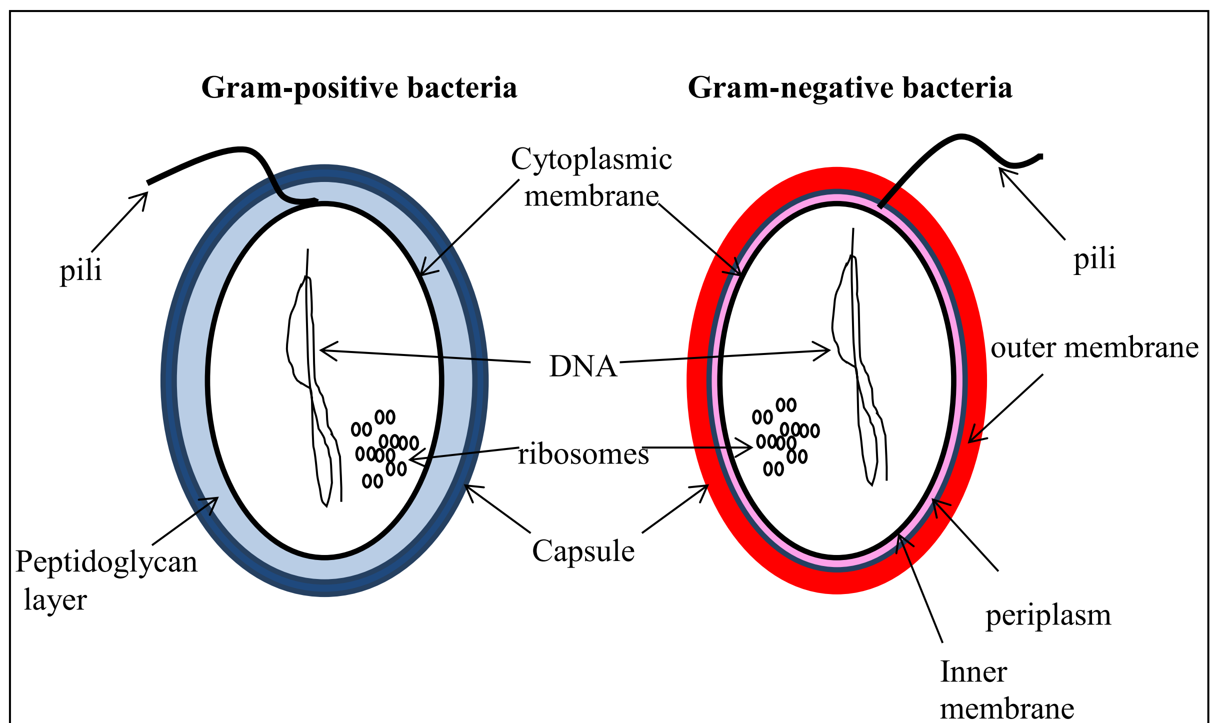


Figure 6. Schematic of Gram-positive and Gram-negative bacteria

The bacterial genome is mostly found in a singular circular chromosome located in the cytoplasm. The bacterial ribosome consists of a 70S ribosome which is significantly different from the eukaryotic 80S ribosome. This difference is exploited by some antimicrobials, such as macrolides, which specifically target

the bacterial ribosome. Mobile bacterial genetic elements, known as plasmids and phages, may also be present in bacterial cells. Plasmids are circular extra-chromosomal molecules of deoxyribonucleic acid (DNA) that are replicated within the cell independently of the main bacterial chromosome. Some smaller plasmids utilise the host cell's own DNA replicative enzymes in order to make copies of themselves, but most plasmids carry genes encoding the ability to replicate themselves. The genes present in plasmids are often responsible for pathogenic characteristics displayed by the host bacterium, such as resistance to antimicrobials. Bacteriophages are viruses that specifically infect bacteria. Like all viruses, phages have a simple structure, consisting of a DNA molecule carrying a small number of genes, surrounded by a protective protein coat. The phage attaches to the outside of the bacterium and injects its DNA into the cell. The DNA molecule is then replicated by specific phage enzymes, followed by protein synthesis. Temperate phages are able to incorporate their DNA into the main bacterial genome in a process called lysogeny, which may then exist for thousands of cell divisions. Both plasmids and phages contribute to bacterial virulence (chapter 1.33).

1.32 Taxonomy

The taxonomy of bacteria is complex, having undergone frequent revisions in the past. Revisions now require the approval of an official international body: the International Committee on Systematic Bacteriology. Bacteria are subdivided in the following successively smaller groups: division, class, order, family, genus and species. A species can be further sub-divided into individual strains. Table 2 describes the taxonomy of three genera containing bacteria that commonly cause keratitis; *Staphylococcus*, *Streptococcus* and *Pseudomonas*.

Bacteria are classified according to their Gram-staining properties and morphological form (chapter 2.121). The two main morphological forms are spheres (cocci) and rods (bacilli). Biochemical reactions, such as activity by the enzymes catalase, coagulase and oxidase (section 2.12), and the ability to utilise different carbon and nitrogen sources for growth, are used to further classify bacteria. Bacteria can also be characterised using molecular approaches, targeting components such as nucleic acids or amino acids. DNA typing and sequencing has further highlighted the heterogeneity of bacteria within their various sub-groups.

Table 2: Taxonomy of the bacterial genera; *Staphylococcus, Streptococcus and Pseudomonas*.

Phylum	*Firmicutes*		*Proteobacteria*
Class	*Bacilli*		*Gammaproteobacteria*
Order	*Bacillales*		*Pseudomonadales*
Family	*Staphylococcaceae*	*Streptococcaceae*	*Pseudomonadaceae*
Genus	*Staphylococcus*	*Streptococcus*	*Pseudomonas*
Species	*S. aureus** *S. epidermidis** *S. hyicus-intermedius* *S. delphini,* *S. lutrae,* *S. pseudintermedius* *S. saprophyticus* *S. auricularis* *S. carnosus* *S. haemolyticus* *S. lugdunensis*	*S. pneumoniae** *S. pyogenes* *S. agalactiae* *Enterococcus**	*P. aeruginosa** *P. alcaligenes* *P. anguilliseptica* *P. argentinensis* *P. borbori* *P. citronellolis* *P. flavescens* *P. mendocina* *P. nitroreducens* *P. oleovorans* *P. pseudoalcaligenes*

* = common causes of keratitis

1.33 How do bacteria cause disease?

Most bacteria live harmlessly in the environment and have nothing to do with living organisms. Furthermore, there are a large numbers of bacteria known as commensals, that live on the surface of humans and animals without causing any impairment of function or disease. A small proportion of bacteria, however, known as pathogens, have the capacity to cause disease.

1.331 Definitions: pathogenicity and virulence

The ability for a bacteria to cause disease is known as its pathogenicity. Virulence is defined as the degree of pathogenicity as indicated by the ability of the bacteria to invade the host and/or the severity of disease outcome. Virulence is dependent on virulence factors; a large array of molecules secreted by pathogens, that enhance their potential to cause disease by enabling host colonization, evasion of host immunity and entry into the host cell. Some virulence factors are chromosomally encoded and intrinsic to the bacteria, whereas others are obtained from mobile genetic elements like plasmids and bacteriophages. Virulence factors encoded on mobile genetic elements spread through horizontal gene transfer, and can convert harmless bacteria into dangerous pathogens.[92] Such factors include bacterial toxins, proteases, cell surface proteins, cell surface carbohydrates and hydrolytic enzymes.[93]

Mechanisms of virulence factors will now be described. Virulence factors particular to *S. aureus* and *P. aeruginosa* will be discussed in detail in chapters 3.12 and 4.12 respectively.

1.332 Evasion of the host immune system

The human host immune system presents a formidable challenge for any invading bacteria. It consists of a non-specific or innate system as well as an adaptive or cell-mediated system.[94-96]

Most infectious agents induce inflammatory responses by activating innate immunity. Local inflammation and the phagocytosis of invading bacteria may also be triggered as a result of the activation of complement on the bacterial cell surface. Macrophages and neutrophils of the innate immune system provide a first line of defence against many bacteria. The innate immune response relies on the recognition of molecules associated with pathogens, such as peptidoglycans and flagella. These are knows as pathogen-associated molecular patterns (PAMPs), which are structures essential for the survival of the pathogen and distinguishable from the host. Upon PAMP recognition, the innate system triggers pro-inflammatory and antimicrobial responses by activating a multitude of intracellular signalling pathways, including adaptor molecules, kinases, and transcription factors.[94] The innate immunity also initiates the adaptive immune responses. The lymphocytes (T and B cells) of the adaptive immune system provide a more robust means of defence, which also provides increased protection against reinfection with the same pathogen via memory.[95] Pathogenic bacteria have various strategies to evade both the innate and adaptive immune systems. Strategies specific to *S. aureus* and *P. aeruginosa* will be described in chapters 3.12 and 4.12 respectively.

1.333 Bacterial adhesion, invasion and toxicity

For bacteria to effectively colonize and cause disease, they must firstly attach to host cells.[97] Most bacteria will only infect hosts that express particular receptors

for bacterial adhesion factors on their cell surface. Bacteria use many types of appendages for adhering to the host surface including, pili, fimbriae and flagella (chapter 1.131). In many cases, the binding structure is a pilus - a long multi-subunit appendage that often has binding specificity in the terminal subunits.

Bacterial invasion occurs either by the production of extracellular enzymes that damage host tissue, or through the modulation of the host response system such as the up-regulation of cytokines. Some virulent bacteria produce proteins that either disrupt host cell membranes or stimulate endocytosis into host cells. These toxins allow the bacteria to enter host cells and facilitate entry into the body across epithelial tissue layers causing tissue damage.[93] Examples of invasive mechanisms include α and γ toxins in *S. aureus* (chapter 3.122) and the type three secretion system and Las B in *P. aeruginosa* (chapter 4.1222)

1.334 Bacteria working in a community; Quorum sensing and biofilms

Inter bacterial communication enables a large number of bacteria to target the host simultaneously. The production of several extracellular virulence factors allows the bacteria to monitor bacterial cell density, communicate with each other and to behave as a population instead of individual cells.[98] This mechanism, called quorum sensing (QS), has been described in many Gram-negative[99] and Gram-positive bacteria.[100] QS is an intercellular signalling process[101] allowing bacteria to migrate to a more inhabitable environment. Molecules known as autoinducers constitute the “language” used for this intercellular communication. When sufficient bacteria are present, autoinducer concentrations reach a threshold level that causes the expression or inactivation of target genes. The effect of QS on the host can be harmonious, as in symbiotic bacteria, or detrimental as in pathogenic bacteria.

QS can lead to individual colonies linking together to form communities that are highly resistant to antimicrobials, known as biofilms. Bacterial biofilms are known to contribute to bacterial keratitis by allowing organisms to persist on contact lenses and contact lens cases despite vigorous antiseptic regimens (chapter 1.1211). Biofilms are also known to cause antimicrobial resistance.[102]

It has also been discovered that there is a universal communication signal synthesized by different bacterial species (inter-species communication), as well that of signals produced by eukaryotic cells (inter-kingdom communication).[103] This may be integral to the formation of multispecies biofilm communities that are important in infection and disease.

1.34 Bacteria causing keratitis

Many bacteria have been implicated in causing keratitis. The Microbiology Ophthalmic Group (MOG) collected 772 bacterial isolates from patients with keratitis from seven ophthalmic-microbiology centres in the UK (London, Birmingham, Bristol, Newcastle, Manchester, Belfast and Liverpool), from 2003 to 2010. See appendix E for members of MOG. Table 3 shows the variability in the proportions of bacteria causing keratitis between the study by the MOG (Sueke et al[104]) and three similar studies set in different geographical locations. For example, the proportion of Gram-positive isolates (57%) was similar to that in the study by Tuft and Matheson[105] in London (56%), but much lower than that in Bourcier et al[2] in Paris (83%) and Bharathi et al[10] in South India (74%). *S. aureus* varied between 8% in France, to 33% in the UK (Tuft and Matheson), and Coagulase-negative staphylococci (CNS) from 17% in India to 48% in France. The most common bacteria isolated by the MOG,[104] Bharathi[10] et al and Bourcier et al[2] were. CNS was not included by Tuft and Matheson,[105] perhaps due to the

uncertainty as to whether this species is pathogenic (chapter 1.3412). The differences between the studies in table 3 may reflect differences in patient population, climate and the prevalence of risk factors such as contact lens use, trauma, or coexistent disease.

Table 3. Bacteria isolated from cases of bacterial keratitis

Study	Sueke[104]	Tuft[105]	Bourcier[2]	Bharathi[10]
Country	UK	UK	France	India
Size of study	n=772	n=1312	n=208	n=1109
Gram-positive bacteria				
total	**57%**	**56%**	**83%**	**74%**
Coagulase negative staphylococci	27%	N/A	48%	17%
Staphylococcus aureus	14%	33%	8%	N/A
Streptococcus	12%	19%	9%	38%
Other Gram-positives*	4%	4%	18%	19%
Gram-negative bacteria				
total	**43%**	**44%**	**17%**	**26%**
Pseudomonas aeruginosa	21%	25%	10%	18%
Enterobacteriaceae	14%	9%	6%	1%
Moraxella spp.	3%	6%	1%	N/A
Haemophilus spp.	1%	2%	N/A	N/A
Other Gram-negatives**	4%	2%	N/A	7%

**Corynebacterium* spp., *Bacillus* spp., *Enterococcus* spp., *Listeria* spp., *Nocardia*.

***Acinetobacter* spp., *Stenotrophomonas maltophilia, Neisseria* spp., *Pasturella* spp., *Aeromonas* spp., *Eikenella* spp., *Agrobacterium* spp., *Alcaligines* spp., *Methylbacterium* spp.

N/A = bacterial species not mentioned in study

1.341 Gram-positive bacteria

The most common Gram-positive bacterial to be isolated from patients with keratitis are from the genera Streptococci and Staphylococci.

1.3411 Streptococci

The genus *Streptococcus* belongs to the phylum Firmicutes and Family *Streptococcaceae* (table 2). The bacteria grow in chains or pairs, which explains the derivation of the genus name; "streptos" meaning twisted chain in Greek. Streptococci possess a typical Gram-positive cell wall containing peptidoglycan and teichoic acid. Lancefield developed a classification system of Streptococci in the 1930s based on isolates containing the same antigenic carbohydrates. The Streptococci can produce exotoxins that damage red blood cells (RBCs) and this is also used to classify the genus. The extent of the lysis can be quantified by examining the discolouration of the agar surrounding colonies. Strains that cause partial lysis of RBCs cause a green discolouration of the surrounding agar and are called α-haemolytic streptococci. Strains that cause full lysis of RBCs lead to clear surrounding agar and these are called β-hemolytic streptococci. When there is no change in the agar colour the isolate is called non-haemolytic. α-haemolytic streptococci include *S. pneumoniae* and *S. viridans* and β-haemolytic streptococci include *S. pyogenes* and *S. agalactiae.*

S. pneumoniae is the most commonly isolated streptococci in patients with keratitis. Using a Gram stain *S. pneumoniae* appears as Gram-positive cocci in pairs (diplococci) and its cell wall contains an antigen which classifies the isolate into Lancefield Group C. The feature of *S. pneumoniae* that is most clearly associated with virulence is the capsular polysaccharide. Laboratory strains that have lost the ability to produce a capsule are not pathogenic. *S. pneumoniae* as

with the other Streptococci have complex nutritional requirements. It requires agar incorporated with 5% sheep blood and incubation in a CO_2 enriched atmosphere.

1.3412 Staphylococci

Staphylococcus was first identified in 1881 as a cause of wound infection by the Scottish surgeon Sir Alexander Ogston.[106] He named it *Staphylococcus* describing the grape-like clusters ("staphyle" in Greek,) he observed under the microscope. He then, in an elegant series of clinical observations and laboratory studies, described staphylococcal disease and its role in sepsis and abscess formation.[107] More than forty species of staphylococci have now been recognised, most of which are not found in humans. Most of those found in humans are harmless, residing on the skin and mucous membranes.

The staphylococci are Gram-positive cocci belonging to the family Micrococcaceae, which encompasses two genera: *Staphylococcus* and *Micrococcus*. Staphylococci are catalase producing and morphologically appear as spherical cells occurring singly in pairs or in clusters. They are non-motile and non-spore forming. Some species in the genus *Staphylococcus* produce the enzyme coagulase, which is otherwise known as clumping factor. This enzyme is used to distinguish between certain isolates in the genus (chapter 2.122). Coagulase-positive species include *S. aureus*, *S. delphini*, and *S. lutrae,* of which only *S. aureus* is a common cause of keratitis.

S. aureus

In 1884 Anton J. Rosenbach, a German surgeon, isolated two strains of staphylococci, which he named for the pigmented appearance of their colonies: *Staphylococcus aureus*, from the Latin aurum for gold, and *Staphylococcus albus*

(now called *epidermidis*), from the Latin albus for white.[108] Humans beings are a natural reservoir of *S. aureus*. It is estimated that around 40% of healthy adults are colonized, with 10 to 20% persistently colonized.[109] *S. aureus* can cause a range of illnesses, from minor skin infections, such as impetigo, furuncles, cellulitis, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis and endocarditis. *S. aureus* is a common cause of keratitis in most studies (table 3).

S. aureus grows easily on routine laboratory culture media such as columbia and blood agar and their optimum growth temperature is 30 °C - 37 °C. Since it is a facultative anaerobe, it has the ability to grow in both aerobic and anaerobic conditions. After 18-24 hours of incubation colonies measuring 4-8 mm are typically seen. Colonies are opaque and may be white, cream or yellow (figure 7), as the extent of pigmentation is a variable trait. The clinical features and virulence factors of *S. aureus* keratitis are discussed in chapter 3.

Coagulase-negative staphylococci

Staphylococci that do not clump when plasma is added are called coagulase-negative staphylococci (CNS). Over 30 species of CNS have been identified. The most clinically relevant species in this group are *S. epidermidis* and *S. saprophyticus*. The best known member of this family and the most common bacterium cultured from the eyelids and conjunctiva is *S. epidermidis*. CNS are considered part of the normal conjunctival flora[110, 111] and, despite being isolated from the corneas of patients with bacterial keratitis, it is often uncertain whether it is a pathogen. Although a recent study established that there was a relationship between clinical outcome and the MIC of ciprofloxacin and ofloxacin against *S. aureus*, such a relationship was not apparent for CNS.[4]

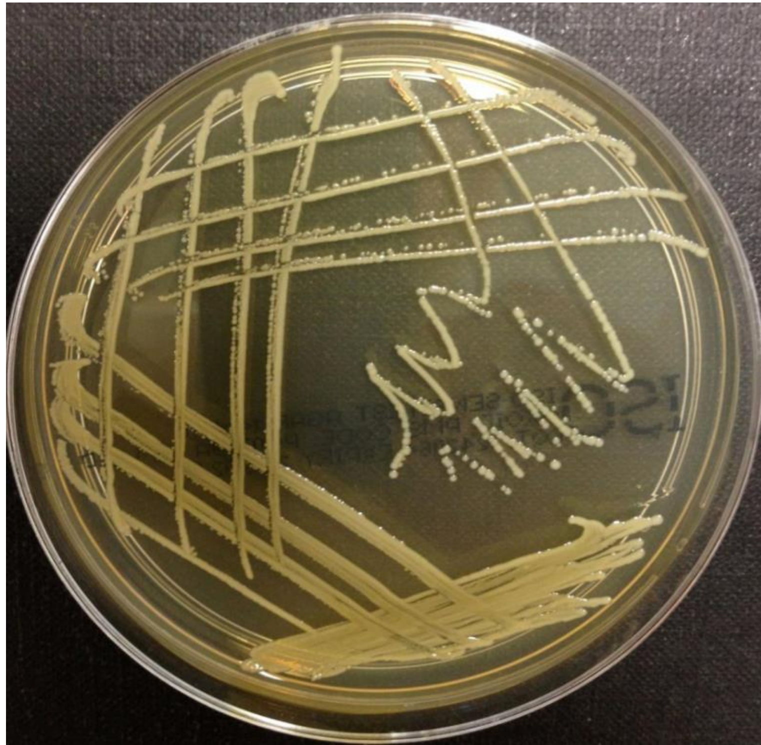


Figure 7. Agar plate showing small pigmented colonies of *S. aureus*

1.342 Gram-negative bacteria

The most commonly isolated Gram-negative bacteria in patients with bacteria keratitis are *P. aeruginosa* and the *Enterobacteriaceae*. Other Gram-negative species have been isolated, for example *Moraxella* spp., *Haemophilus* spp. *Acinetobacter* spp., *Stenotrophomonas maltophilia*, *Neisseria* spp., *Pasturella* spp., *Aeromonas* spp., *Eikenella* spp., *Agrobacterium* spp., *Alcaligines* spp., and *Methylbacterium* spp.

Gram-negative bacteria can be subdivided into two large groups, the fermenters and non-fermenters. A bacterium that can metabolize glucose or other carbohydrates only in the presence of oxygen is defined as a non-fermenter. The non-fermenters account for approximately 15% of all Gram-negative bacilli cultured from clinical specimens and include the species *P. aeruginosa*.[112]

Gram-negatives can also be subdivided into oxidizers; bacteria that can oxidize carbohydrates, and non-oxidizers that cannot. Most of the non-fermenters, such as *P. aeruginosa*, are oxidase-positive. Non-fermentative Gram-negative bacilli are ubiquitous in the environment. They are particularly prevalent in moist environments and hence thrive in contact lens cases.[113] Non-fermenters can also withstand detergents such as clorhexidine and ammonium compounds. They rarely live as part of the normal host microbial flora and are considered to be opportunistic, causing infections only in individuals who are immunocompromised, or whose natural defences are breached. Additional features can differentiate this group of non-fermenters: motility, pigmentation and the lack of ability to grow on certain media.

1.3421 *Pseudomonas*

The genus *Pseudomonas* was first described by Migula in 1894.[114] It is one of the most diverse and ubiquitous bacterial genera whose species have been isolated in all types of environments for example; plant matter, water, minerals, soil, animals and humans. The taxonomy was reclassified in 2000, according to its 16S RNA genotype.[115]

P. aeruginosa is the most common cause of human infection from the genus *Pseudomonas* (table 2). This species is implicated in various life threatening infections and it is a frequent cause of keratitis. *P. aeruginosa* has simple growth requirements and can use a variety of compounds for nutrition. This explains the presence of *P. aeruginosa* infections associated with homemade saline solution and soft contact lenses.[116] *P. aeruginosa* has a growth temperature range of 5-42 °C and can produce at least 6 colonial types after 24 hours of aerobic incubation on nutrient agar. The most common type are large, oval,

mucoid colonies, which can be surrounded by serrated growth (figure 8). Colonial variation from one type to another does not necessarily indicate the presence of more than one strain. *P. aeruginosa* is a strict aerobe and is oxidase and catalase positive. The characteristic blue-green appearance of the organism in culture is due to the mixture of pyocyanin (blue) and pyoverdine (yellow). Some strains produce other pigments, such as pyorubin (red) or pyomelanin (brown). Clinical features and virulence factors of *P. aeruginosa* keratitis are discussed further in chapter 3.

1.3422 *Enterobacteriaceae*

The *Enterobacteriaceae* are a large family of Gram-negative bacilli that can be differentiated from *Pseudomonas* as they are oxidase negative. *Bergey's Manual of Systemic Bacteriology* describes 176 species in the *Enterobacteriaceae* family. Species that have been implicated in keratitis include: *Serratia* spp, *Klebsiella* spp., *Citrobacter* spp., *Proteus* spp., *Escherichia coli*, *Enterobacter* spp., *Morganella morganii* spp. and *Pantoea* spp. Colonial morphology does not readily identify the particular *Enterobacteriaceae* species, except for *Klebsiella* that produce large mucoid colonies. Definitive identification depends on biochemical reactions and serological antigenic structures that differ between species (chapter 2.123).

Figure 8: *P. aeruginosa* colonies on agar plate. Circular raised mucoid colonies stained with pyoverdine green pigment.

1.4 Thesis aims

The clinical outcome of patients with bacterial keratitis varies considerably. Patients with mild disease have a complete resolution of symptoms after a short course of topical antimicrobial treatment, with no long term visual sequelae whereas a minority of patients may require prolonged treatment and are still left with permanent visual loss. There are three main factors, the bacteria, treatment and the host, that interplay and determine the outcome of bacterial keratitis. The aim of this thesis is to investigate the first two of these factors; firstly the characteristics and virulence of the two major species causing keratitis; *S. aureus* and *P. aeruginosa* and secondly the efficacy and susceptibility of antimicrobials, in particular the potentially novel antimicrobial meropenem.

CHAPTER 2: GENERAL METHODS

2.1 Bacteria

2.11 Collection of samples

Bacterial isolates used to investigate virulence factors (Chapters 3.3 and 4.3) and antimicrobial susceptibility (Chapter 5.3), were collected by the MOG from patients presenting with keratitis from seven ophthalmic-microbiology centres in the UK.

Samples were collected by scraping the corneal ulcer using a surgical blade (no. 11 Baird Parker surgical blade; Swann Morton Ltd., Sheffield, UK), with care taken to avoid touching any other part of the eye or its adnexa (figure 9). One blade was streaked onto a glass slide. In three centres only one further scrape was taken which was placed into BHI broth (Oxoid, UK). In the remaining four centres further scrapes were placed directly onto agar plates.

The plates and broth were incubated for 18-24 hours at 37 °C under both aerobic and enriched CO_2 (5%) atmospheric conditions in the local laboratory. If bacteria were identified, colonies were stored on sterile beads (Protect Beads; TSC Ltd., Heywood, Lancashire, UK) and sent to the University of Liverpool where they were stored at -80 °C prior to subsequent analysis.

2.12 Identification of bacteria

Beads were thawed and streaked onto agar under aseptic conditions and incubated in the conditions described in table 4. See appendix A for details of preparation of agar. After 18-24 hours of incubation, colonies were observed. The following tests were performed where necessary to confirm the identity of the bacteria.

(a)

(b)

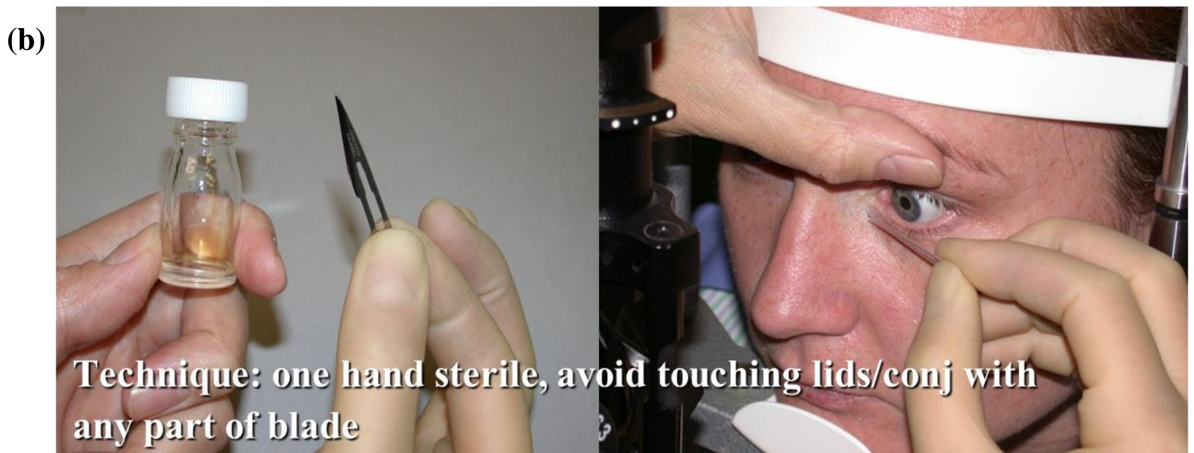


Figure 9: Photograph demonstrating (a) the equipment needed for corneal scrape and (b) the set up and method of corneal scrape

Table 4: Incubation conditions of bacteria cultured in patients with keratitis.

Organisms	Media	Incubation conditions
Enterobacteriaceae	ISA	35-37 °C in air
Acinetobacter spp.	ISA	35-37 °C in air
Pseudomonas spp.	ISA	35-37 °C in air
staphylococci	ISA	35-37 °C in air
α-Haemolytic streptococci	ISA+5% horse blood	35-37 °C in 4-6% CO_2
β-Haemolytic streptococci	ISA+5% horse blood	35-37 °C in air
Streptococcus pneumoniae	ISA+5% horse blood	35-37 °C in 4-6 % CO_2
Haemophilus spp.	ISA+5% horse blood	35-37 °C in 4-6 % CO_2

ISA = Iso-Sensitest agar

2.121 Gram stain

Background: The Gram stain, developed in 1884 by Hans Gram,[117] can differentiate bacteria into two distinct groups based on their cell wall properties. The iodine used in a Gram stain is a mordant and is retained by the thick cell wall in Gram-positive bacteria resulting in the observed purple/blue colour. Gram-negative bacteria do not retain the iodine complexes, and a pink/red colour is the result of the safranin counterstain.

Method: Freshly grown colonies were removed from the agar plate and placed onto a slide. The slide was briefly heated with a flame to fix the isolate to the slide. The slide was flooded with 2% w/v crystal violet and left for 30 seconds. 1% w/v Lugol's iodine was applied to the tilted slide to wash away the stain followed by a further application of iodine. After 30 seconds the iodine was washed off with 100% ethanol until the colour ceased to run out of the isolate.

The slide was rinsed with water and 0.1% safranin was poured on. After 2 minutes the slide was washed with water and blotted dry. Bacterial cells appearing purple/blue on microscopy were considered to be Gram-positive and pink/red were considered Gram-negative. Further biochemical tests were performed depending on the Gram stain result.

2.122 Gram-positive isolates

2.1221 Catalase test

Background: The catalase test was used to differentiate between the two commonest Gram-positive cocci, streptococci and staphylococci, which have similar colonial appearance. As staphylococci contain the enzyme catalase and streptococci do not, the catalase test is a simple method to distinguish these two species. Catalase causes hydrogen peroxide to produce oxygen and water, which explains the formation of bubbles in catalase positive bacteria.

Method: 0.2 mL of hydrogen peroxide solution was placed into a tube. The colony in question was collected from the agar plate with a sterile loop, which was rubbed on the inside wall of the tube above the hydrogen peroxide solution. The bottle was tilted to allow the hydrogen peroxide solution to cover the colony. The presence of vigorous bubbling within 10 seconds was considered a positive result. Positive control *S. aureus* NCTC 6571 and negative control *S. mitis* NCTC 10712 were used.

2.1222 Coagulase test

Background: The coagulase test was used to differentiate the Staphylococci into two main groups: Coagulase-positive staphylococci and Coagulase-negative

staphylococci (CNS). The vast majority of Coagulase-positive staphylococci isolated in humans are *S. aureus*. Coagulase (or clumping factor) bound to the cell wall of Coagulase-positive staphylococci, cause cell agglutination when fibrinogen from plasma is added.

Method: The test strain was emulsified into a homogenous suspension in a drop of distilled water on a slide. A 10 µl loop of the rabbit plasma was gently mixed with the suspension and the presence of visible clumping within 10 seconds was called a positive result. Positive control *S. aureus* NCTC 6571 and negative control *S. haemolyticus* NCTC 42764 were used.

2.123 Gram-negative isolates

2.1231 Oxidase test

Background: *P. aeruginosa* isolates do not always produce the characteristic pigmentation associated with the bacteria and may be mistaken for an *Enterobacteriaceae*. Unlike the *Enterobacteriaceae* however, *P. aeruginosa* possess the enzyme cytochrome oxidase, which catalyses the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The oxidase test utilises the reagent N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride, which acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue in oxidase positive isolates.

Method: Freshly grown colonies were taken with a sterile loop and rubbed onto an impregnated oxidase test strip (Thermo Scientific, UK). A blue colour within

10 seconds indicated bacterial oxidase production and was considered to be a positive result.

2.1232 API test for *Enterobacteriaceae*

Background: Oxidase negative Gram-negative bacilli were presumed to be from the *Enterobacteriaceae* family. An Analytical Profile Index (API) was used to identify the species of *Enterobacteriaceae*. The API 20E system (bioMérieux, UK) was used which consists of a plastic strip of 20 individual, miniaturized tests tubes each containing a different reagent used to determine the metabolic capabilities and the genus and species (table 5).

Method: One freshly grown colony was taken from a culture plate and placed into 5ml sterile water. Each tube in the API-20E strip (figure 10) was inoculated with the bacterial suspension. Some of the tubes were completely filled (tests CIT, VP and GEL), whereas others were topped off with mineral oil to maintain anaerobic conditions (tests ADH, LDC, ODC, H2S, URE). The strip was incubated in air for 18-24 hours at 37 °C and analysed for specific colour changes (table 5). Interpretation of the 20 reactions, was converted to a seven-digit code, which was entered online to receive a presumed species identification (https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin).

Table 5: Components of Analytical Profile Index test to determine *Enterobacteriaceae* species

Test	**Substrate**	**Reaction tested**	**-ve result**	**+ve result**
ONPG	ONPG	beta-galactosidase	colourless	yellow
ADH	arginine	arginine dihydrolase	yellow	red/orange
LDC	lysine	lysine decarboxylase	yellow	red/orange
ODC	ornithine	ornithine decarboxylase	yellow	red/orange
CIT	citrate	citrate utilization	pale green/yellow	green/blue
H2S	Na thiosulfate	H2S production	colourless/grey	black deposit
URE	urea	urea hydrolysis	yellow	red/orange
TDA	tryptophan	deaminase	yellow	brown-red
IND	tryptophan	indole production	yellow	red
VP	Na pyruvate	acetoin production	colourless	pink/red
GEL	charcoal gelatin	gelatinase	no black diffusion	black diffuse
GLU	glucose	fermentation/oxidation	blue/blue-green	yellow
MAN	mannitol	fermentation/oxidation	blue/blue-green	yellow
INO	inositol	fermentation/oxidation	blue/blue-green	yellow
SOR	sorbitol	fermentation/oxidation	blue/blue-green	yellow
RHA	rhamnose	fermentation/oxidation	blue/blue-green	yellow
SAC	sucrose	fermentation/oxidation	blue/blue-green	yellow
MEL	melibiose	fermentation/oxidation	blue/blue-green	yellow
AMY	amygdalin	fermentation/oxidation	blue/blue-green	yellow
ARA	arabinose	fermentation/oxidation	blue/blue-green	yellow

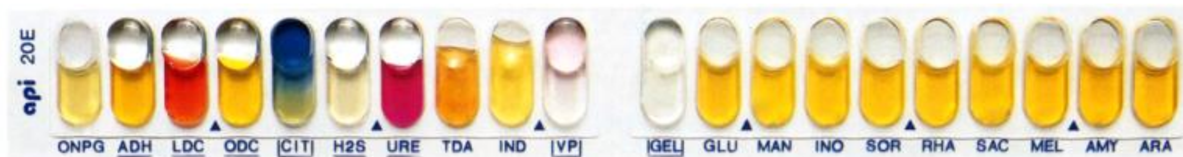


Figure 10: API20E strip to identify *Enterobacteriaceae* species

2.2 Antimicrobial testing

2.21 Antimicrobial susceptibility

Background: The response of a bacterium to an antimicrobial can be quantified by determining the lowest antimicrobial concentration required to inhibit overnight bacterial growth, known as the minimum inhibitory concentration (MIC). The broth dilution method is the traditional method of determining the MIC, however, as this method is labour intensive, the E-Test method is preferred. E-Tests are inert, non-porous plastic strips measuring 5 x 60 mm, containing an exponential gradient of the dried and stabilised antimicrobial (figure 11). One side of the strip is calibrated with MIC values in mg/ml and a two-letter code designates the identity of the antimicrobial. When an E-Test is applied onto an inoculated agar plate, there is an immediate release of the agent from the plastic surface into the agar plate. When bacterial growth is visible, following incubation, a symmetrical inhibition ellipse centred along the strip is seen. The zone edge intersection with the E-Test is the MIC value.

Method: Antimicrobial susceptibility testing was performed on bacteria collected by the MOG using E-Tests, according to British Society of Antimicrobial Chemotherapy (BSAC) methods.[118] Frozen beads were thawed and incubated on agar using conditions as described in table 4. Bacterial suspensions were prepared by transferring a loop of colonies directly from the plates into tubes containing sterile distilled water. The suspension was made to a 0.5 McFarland standard using a calibrated spectrophotometer at a wavelength of 500 nm. The bacterial suspension was diluted with sterile water; 1 in 10 for *S. aureus*, 1 in 100 *P. aeruginosa* and suspensions were used within 15 minutes of preparation. A sterile cotton-wool swab was dipped into the diluted suspension and the excess liquid

removed by turning the swab against the side of the tube. Susceptibility media was inoculated by evenly spreading the swab over the entire surface of the plate in three directions. E-Tests (AB Biodisk; Solna, Sweden) were placed centrally on the inoculated plates. Following incubation in air at 37 °C for 18 to 24 hours, MICs were calculated by determining the point of intersection of zone of inhibition with E-Test strip (figure 11). Tiny colonies at the edge of the zone were ignored and colonies growing within the zone of inhibition were subcultured and identified and the test repeated if necessary. Plates with too heavy or too light growth were also repeated. Quality control strains *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *S. pneumoniae* ATCC 49619 were included.

Figure 11: Teicoplanin E-Test on agar plate inoculated with *S. aureus.* MIC calculated at 8 mg/L; point of intersection of zone of inhibition with E-Test strip (indicated with arrow).

2.22 Identification of meticillin resistance

To determine whether a staphylococci isolate was resistant to meticillin, a bacterial inoculum was prepared and spread onto Iso-Sensitest (ISA) agar as described above. A disc containing 10 µg cefoxitin was placed on the surface of inoculated agar and plates were incubated in air at 35 °C for 18-20 hours. The zone of inhibition surrounding the disc was carefully measured to the nearest millimetre with a ruler. Zones of inhibition were considered to be susceptible if ≥ 22 mm diameter and resistant if ≤21 mm diameter, as per BSAC guidlines.[118] Control strains were used: *S. aureus* ATCC 25923 (susceptible) and *S. aureus* NCTC 12493 (resistant).

2.3 Molecular techniques

2.31 Deoxyribonucleic acid extraction

Two methods of deoxyribonucleic acid (DNA) extraction were used to investigate virulence factors. Firstly, the Wizard Genomic DNA purification kit was used in *P. aeruginosa* (chapter 4.3), as suggested by the Clondiag Array Tube manufacturer's instructions. Secondly, the more time efficient QIASymphony method was used for *S. aureus* (chapter 3.3).

Genomic DNA was extracted from *S. aureus* isolates using QIASymphony SP (Qiagen, UK), following manufacturer's instructions. 1 ml of overnight culture was pelleted and washed in 500 µl phosphate buffered saline (PBS) and resuspended in Lysis buffer (20 mM Tris·HCl; 2 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0; 1.2% (v/v) Triton) containing 20 µg/ml lysostaphin. Cells were incubated at 37 °C for 1 hour before the addition of 20 µl proteinase K and

200 µl Buffer AL (Qiagen, UK) followed by incubation at 56 °C for 30 min. The lysate was transferred to QIASymphony compatible tubes and DNA was purified using the Pathogen Complex 200 protocol. Purity and concentration of isolated DNA was determined spectrophotometrically, by measuring the absorbance at 260 nm and 280 nm (NanoDrop Technologies; Abtech International, UK). Preparations which had 260/280 nm and 260/230 nm ratios between 1.8 and 2.0 were considered to be of sufficient purity.

Genomic DNA was extracted from *P. aeruginosa* isolates, using the Wizard Genomic DNA purification kit (Promega, UK). Bacterial cultures were grown overnight in Luria-Bertani broth (LB) at 37 °C in air (appendix A for LB broth methods). Bacterial cells were pelleted by centrifuging 1 ml of culture for 2 minutes at 16,000×g. 600 µl Nuclei Lysis Solution was added followed by incubation for 5 minutes at 80°C. 3 µl of RNase Solution was added, followed by incubation at 37 °C for 60 minutes. 200 µl of Protein Precipitation Solution was added followed by incubation on ice for 5 minutes. After centrifuging at 16,000 ×g for 3 minutes, the supernatant was transferred to a tube containing 600 µl of isopropanol. The mixture was centrifuged and the supernatant removed. 600 µl of room temperature 70% ethanol was then added followed by centrifuge for 2 minutes at 16,000 ×g. Ethanol was removed and the pellet left to air-dry 15 minutes. The DNA pellet was rehydrated in 100 µl of Rehydration Solution for 1 hour at 65 °C. Samples were checked for purity and concentration of DNA spectrophotometrically, as described above.

2.32 Nucleic acid amplification

2.321 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) amplification was used to determine the presence or absence of virulence factor genes in *S. aureus* and *P. aeruginosa* from extracted DNA. The PCR mastermix comprised BioMix red (Bioline, UK), magnesium chloride 3.0 mM, forward and reverse primers (see table 6 for primer details) and DNA. Chapters 3 and 4 describe individual PCR conditions.

2.322 Gel electrophoresis

Agarose electrophoresis powder (Quiagen, UK) was added to 0.5x tris-borate-ethylenediaminetetraacetic acid (TBE) to a concentration of 1% w/v. See appendix B for methods of making TBE. The mixture was heated in a microwave 1-3 minutes until the powder was completely dissolved. The solution was cooled down for 5 minutes and ethidium bromide was added, to make a final concentration of 0.5 µg/mL. The agarose gel was slowly poured into a gel tray with a well comb in place. Air bubbles were pushed away from the well comb with a pipette tip. The gel was then let to sit at room temperature until it completely solidified (20-30 minutes). The gel was placed into an electrophoresis unit was and was bathed in 0.5x TBE. 50 bp DNA ladder (Invitrogen, UK) (see appendix B3) was added into the first lane of the gel and samples containing the master mix and DNA were loaded into the subsequent wells. Gels were run at 100 V until the dye line was approximately 75% of the way down the gel (about 1-1.5 hours). DNA fragments (seen as bands) were then visualised and photographed using ultraviolet light.

Table 6. Oligonucleotide primers used in PCR for (a) amplification of: *Staphylococcus g*enus-specific 16S rRNA, *lukSF-PV* and *mecA*, (b) full gene sequencing of *lukSF-PV* and (c) MLST of *lukSF-PV* +ve *S. aureus* isolates.

	Gene	Primer	Sequence (5'-3')
(a)	16s rRNA	Staph756F	AACTCTGTTATTAGGGAAGAACA
		Staph750R	CCACCTTCCTCCGGTTTGTCACC
	lukSF-PV	Luk-PV1	ATCATTAGGTAAAATGTCTGGACATGATC
		Luk-PV2	CAGCATCAAGTGTATTGGATAGCAAAAGC
	mecA	MecA1	GTAGAAATGACTGAACGTCCGATAA
		MecA2	CCAATTCCACATTGTTTCGGTCTAA
(b)		PVL1F	GGTGATGGCGCTGAGGTAGTCAAA
		PVL1R	CTGTATGATTTTCCCAATCAACTTC
	lukSF-PV	PVLint2F	CAACTGCAACATCAGATTCCGATAAG
		PVLint2R	CAAATTCACTTGTATCTCCTGAGCC
		PVLint3F	GGGACCATATGGCAGAGATAGTTATC
		PVLint3R	GTATTGGAAAGGCCACCTCATTGC
(c)	*Carbomate kinase*	arcC-Up	TTGATTCACCAGCGCGTATTGTC
		arcC-Dn	AGGTATCTGCTTCAATCAGCG
	Shikimate dehydrogenase	aroE-Up	ATCGGAAATCCTATTTCACATTC
		aroE-Dn	GGTGTTGTATTAATAACGATATC
	Glycerol kinase	glpF-Up	CTAGGAACTGCAATCTTAATCC
		glpF-Dn	TGGTAAAATCGCATGTCCAATTC
	Guanylate kinase	gmk-Up	ATCGTTTTATCGGGACCATC
		gmk-Dn	TCATTAACTACAACGTAATCGTA
	Phosphate acetyltransferase	pta-Up	GTTAAAATCGTATTACCTGAAGG
		pta-Dn	GACCCTTTTGTTGAAAAGCTTAA
	Triosephosphate isomerise	tpi-Up	TCGTTCATTCTGAACGTCGTGAA
		tpi-Dn	TTTGCACCTTCTAACAATTGTAC
	A acetyltransferase	yqiL-Up	CAGCATACAGGACACCTATTGGC
		yqiL-Dn	CGTTGAGGAATCGATACTGGAAC

2.4 Cell culture

Experiments using cell culture (chapter 5.331) were performed aseptically in a Class II biological cabinet. Cultures of Human Keratocytes (HKs) were established from the rims of donor corneo-scleral discs that had been used for corneal transplantation using cells from passages 5 to 10 as previously described.[119] Human corneal epithelial cells (HCEs), immortalized by the SV40 virus, were kindly provided by Dr Araki-Sasaki (Kiniki University, Hyogo, Japan).[120] Cryopreserved HKs and HCEs were thawed and grown in a designated tissue culture incubator in 5% CO_2 at 37 °C. HCEs and HKs were maintained at 37 °C in 5% CO_2 in a mixture of Dulbecco's Modified Eagle Medium (DMEM) and Nutrient Mixture F-12 (Sigma, Dorset, UK) supplemented with 10% fetal calf serum (FCS) (Biosera, East Sussex, UK), 1% L-glutamine and 1% penicillin & streptavidin (Sigma, Dorset, UK). When cells became sub-confluent (70-80% confluent) cells were split (passaged) to allow for further cell growth as follows. Trypsin was added to the culture-dish to loosen the cells. Media (with FCS) was added to the cells to quench trypsin reaction. Cells were then removed and centrifuged at 1000 rpm for 5 minutes. The pellet was re-suspended in 10 ml fresh media and the cell culture was split into separate dishes or flasks, and topped up with fresh media. Media was replaced every other day.

CHAPTER 3 *STAPHYLOCOCCUS AUREUS*

3.1 Introduction

S. aureus is frequently implicated in bacterial keratitis (Table 3).[104] Out of 772 isolates collected by the MOG from patients with keratitis 14% were *S. aureus.*

3.11 Clinical features

S. aureus associated keratitis is typically insidious in onset.[121] Symptoms of a red, painful eye may have a gradual onset over a few weeks. On examination a localised small grey-white ulceration with clear margins is frequently seen. There is usually only minimal surrounding epithelial oedema and stromal infiltrate. Typically the lesion is in the periphery of the cornea, although it can develop in a more central location. A moderate anterior chamber reaction may be present. Long standing staphylococcal ulcers penetrate deep into the stroma producing intra-stromal abscesses and occasionally perforation. Multiple satellite stromal micro-infiltrates may also be seen.

Blepharitis is a common predisposing feature for *S. aureus* keratitis. It is characterized by thickened eyelid margins with telangiectasia, scaling and crusting (figure 12).[122] Loss of eyelashes and corneal involvement, including punctate epithelial erosions, marginal infiltrates, and neovascularization, may occur. MGD is characterized by terminal duct obstruction and/or changes in the glandular secretion. MGD may result in a poor tear film and ocular surface disease. Patients with MGD are frequently noted to have coexisting acne rosacea or seborrhoeic dermatitis. Chronic inflammation may be punctuated by acute exacerbations that lead to the development of corneal ulceration.[123, 124]

Scarring, CoNV and exudation from previous episodes of keratitis is suggestive of possible endogenous sources of bacteria particularly *S. aureus* from the eye lids or nares.

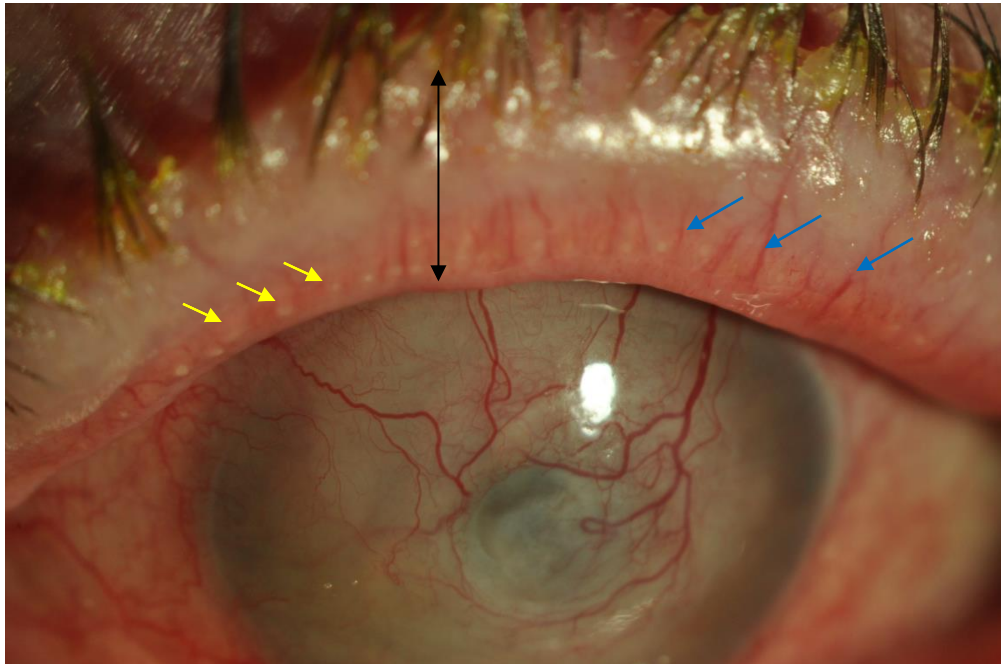


Figure 12. Picture of keratitis associated with *S. aureus.* Features; severe central corneal thinning and aggressive corneal neovascularisation. Note blepharitis; thickened lid margin (black arrow), blocked and retroplaced meibomian gland orifices (yellow arrows), lid margin vascularisation (blue arrows).

3.12 Virulence factors

Humans beings are a natural reservoir for *S. aureus* with 20% persistently colonised with the bacteria in their anterior nares and throat.[109,125] Singer et al found 3% of conjunctival swabs taken from healthy eyes grew *S. aureus* in culture.[126] Whether an infection is contained or spreads is dependent on a complex interplay between *S. aureus* virulence determinants and host defence mechanisms. *S. aureus* has an extraordinary array of virulence factors that allows it to survive

extreme conditions within the host. These factors are controlled by a complex regulatory network in response to cell density, energy availability and environmental signals. Four distinct signal transduction systems have been described; *agr*, *sae*, *srr* and *arl*,[127] of which *agr* is the most well known due to its regulation of QS.[128] *S. aureus* virulence factors (summarised in table 7), will now be described. Antimicrobial resistance is described in chapter 5.

Table 7. Summary of *S. aureus* virulence factors

Pathogenic function	Virulence factor
Bacterial survival in host	
Neutrophil inhibition	Phenol soluble modulin, chemotaxis inhibitory protein, extracellular adherence protein.
Opsonophagocytosis inhibition	Antioxidants: catalase, staphyloxanthin, superoxide dismutase Bacterial surface capsule, clumping factor A, protein A, multiple complement inhibitors
Evasion of adaptive immunity	Enterotoxins (T-cell inhibition), protein A (B-cell inhibition)
Bacterial nutrition	iron acquisition program; aureochelin and staphyloferrin;
Bacterial invasion and host destruction	
Adherence	Staphylococcal protein A, fibronectin & collagen binding protein, clumping factor
Cytotoxicity	α-haemolysin, β-haemolysin, enterotoxin b and c, toxic shock syndrome toxin 1, exfoliative toxins, epidermolytic toxins A and B, α and γ toxins, Panton–Valentine leukocidin
Tissue destruction	protease, lipase, and hyaluronidase
Antimicrobial resistance	
Meticillin resistance	*mecA*

3.121 Bacterial survival within the host

The success of *S. aureus* as a pathogen is due in part to its ability to overcome the host's immune system. One of the key host cells in pathogen defence are neutrophils (chapter 1.332) and *S. aureus* has many mechanisms to evade destruction by them. *S. aureus* secretes chemotaxis inhibitory protein and extracellular adherence protein which block neutrophil recognition of chemotactic factors[129] and binding to endothelial adhesion molecule ICAM-1.[130] Reactive oxygen species produced by neutrophils are neutralized by the deployment of the antioxidant enzymes known as catalases and superoxidase dismutase produced by *S. aureus*.[131] *S. aureus* also secretes a specific toxin; phenol soluble modulin which induces neutrophil cytolysis.[132] *S. aureus* expresses a surface capsule, clumping factor A, protein A and at least four complement inhibitors, which minimise opsonisation by complement and antibodies.[131]

A severe bacterial infection normally induces the host to mount an adaptive immune response within 7 to 10 days. Studies have shown that *S. aureus* produces enterotoxins that can alter T cell functions,[133] and protein A that suppresses the host B cell response.[134]

In addition to evasion of host immune defence, the survival of *S. aureus* within the human host is dependent on successful acquisition of nutrients, particularly iron. *S. aureus* secretes the high affinity iron-binding siderophores aureochelin and staphyloferrin, to achieve this. The bacteria also initiates transcription of an iron acquisition programme upon sensing low iron levels, that allows capture and processing of iron within the cell.[135] Biofilm formation (chapter 1.33) is another *S. aureus* virulence mechanism. This allows the bacteria

to persist on foreign material such as contact lenses[136] and resist host defences and antimicrobials.

3.122 Bacterial adherence and invasion

Bacterial adhesive proteins are an essential component for *S. aureus* to cause disease. Corneal adhesion of *S. aureus* is thought to be due to fibronectin-binding protein[137] and collagen-binding adhesin protein.[138]

The tissue damage resulting from *S. aureus* infection has been attributed to various toxins. α toxin, a cytolytic toxin produced by nearly all *S. aureus* isolates, causes cell death of macrophages and lymphocytes by forming pores in their cell membranes.[139] The toxin also alters platelet morphology, which may contribute to increased thrombotic events associated with *S. aureus* sepsis. α toxin has also been found to be an important virulence factor in animal models of keratitis.[140, 141]

Other toxins that are produced by *S. aureus* that contribute to virulence include γ toxin, S-type protein, F-type protein and Panton-Valentine Leukocidin (PVL).[142] However, the role of these toxins in the pathogenesis of keratitis has yet to be fully elucidated.

3.123 Panton Valentine Leukocidin

In 1894 van de Velde discovered the leukocidal property of some *S. aureus* isolates.[143] Doctors Panton and Valentine attributed this to the production of a toxin which was named Panton-Valentine Leukocidin (PVL).[144] PVL is a phage-encoded bicomponent toxin that consists of the two polypepetides lukS-PV and lukF-PV.[145] The genes encoding PVL, *lukS-PV* and *lukF-PV*, have been found to reside in the genomes of 6 phages: ΦSa2958, ΦSa2MW, ΦPVL, Φ108PVL, ΦSLT, and ΦSa2USA.[146] Despite the number of different PVL phages, the genes

that encode PVL have been shown to be relatively conserved. Studies have found LukS-PV and LukF-PV to bind to neutrophils, monocytes and macrophages but not to lymphocytes.[147, 148] Following the binding of monomers of LukS-PV and LukF-PV, further monomers attach to the cell wall forming a heptameric structure that forms a pore in the host cell surface.[145, 149] This pore formation results in leukocyte cell death and the release of inflammatory cytokines.[148]

A review of *S. aureus* infections reported to the French Reference Centre for Staphylococcal Toxaemia identified eight cases of severe community-acquired pneumonia caused by *S. aureus* strains carrying the PVL gene, six of which were fatal.[150] Lina et al[151] found that in 176 cases of *S. aureus* infection, PVL genes were strongly associated with furunculosis (93%), severe necrotic haemorrhagic pneumonia (85%), cellulitis (55%), cutaneous abscess (50%) and osteomyelitis (23%). PVL-encoding genes, however, were not detected in strains responsible for other infections, for example, infective endocarditis, mediastinitis, hospital-acquired pneumonia, urinary tract infection, and enterocolitis, or in those associated with toxic-shock syndrome. Table 8 describes the prevalence of the *lukSF-PV* locus in eight studies.[152-157] Rates range from 2.1% in a Dutch study[154] to 51% in an Indian study (table 8).[157] Differences in prevalence between studies may reflect differences in clinical sources of infection or different geographical locations.

Table 8. Reported rates of *lukSF-PV* carriage in *S. aureus* isolates worldwide

	Source of *S. aureus* isolates	**n**	***lukSF-PV* +ve (%)**	**Ref**
Thailand	Hospital patients range of disease	270	49	152
USA	Eurofins Medinet database	1055	36	153
Netherlands	Nasal samples from healthy patients	829	0.6	154
Netherlands	Blood cultures from patients with invasive disease	146	2.1	154
France	Range of samples from hospital patients	309	2.1	155
Singapore	Range of samples from hospital patients	204	9.8	156
India	Patients with various eye infections	33	51.5	158
UK	Reference Unit (not all from disease)	515	1.6	159

The involvement, however, of PVL in the virulence of *S. aureus* is equivocal and its link with clinical outcome remains uncertain. *In vivo* studies have produced conflicting data. Murine models of *S. aureus* infection have shown that the absence of PVL results in an increase in virulence,[160-163] whereas studies in rabbits indicate that the presence of PVL increases the virulence of *S. aureus*.[164-166] These discrepancies could however be attributed to differences in the immunology of the models.[166] Mouse neutrophils are insensitive to the effects of PVL whereas rabbit and human neutrophils both have sensitivity. There is also no consensus on the effect of *lukSF-PV* on clinical outcome in patients with *S. aureus* infection. Some studies suggest that PVL is not associated with clinical outcome or that the presence of PVL can reduce virulence,[160, 167-169] whereas other studies have found a correlation between PVL carriage and diseases invasiveness.[170-173]

In terms of antimicrobial treatment, the most common isolate responsible for outbreaks of community acquired meticillin resistant *S. aureus* (MRSA) infections

in the USA, was the PVL+ve strain USA300. The epidemic of the USA300 strain has not yet been seen in the UK, where the vast majority of MRSA isolates are *lukSF-PV*-ve.[174] PVL+ve *S. aureus* isolates are susceptible to most other antimicrobials used to treat staphylococcal infections, including trimethoprim-sulfamethoxazole, glycopeptides, linezolid and the fluoroquinolones.[174-176] One particular sequence type (ST80), however, has been found to be resistant to tetracycline and fusidic acid.[175, 176]

The potential involvement of PVL as a virulence factor has led to the investigation of its relevance in infectious diseases of the eye and its adnexa. Rutar et al[177] described a case series of 9 patients with severe ocular infection (including cases of orbital cellulitis and endophthalmitis) caused by the USA300 *S. aureus* strain that is known to carry *lukSF-PV.* More recently, Nadig et al[158] reported 33 cases of ophthalmic infections caused by *S. aureus* taken from two centres in India. They found 17 (52%) of the isolates were *lukSF-PV*+ve, while 5 of the 9 (56%) keratitis isolates were *lukSF-PV*+ve. Four of the *lukSF-PV*+ve keratitis isolates were resistant to fluoroquinolones, but outcome data was not reported. There are no reported studies, however, that have looked for an association between the *lukSF-PV* genotype and clinical outcome in keratitis caused by *S. aureus.*

3.2 Chapter aims

This chapter aims to investigate the prevalence and type of *lukSF-PV* in *S. aureus* isolates taken from patients with keratitis in the UK. An association will be made between the presence of *lukSF-PV* and clinical outcome in patients with keratitis associated with *S. aureus*.

3.3 Methods

3.31 Collection of isolates and determining MICs

Clinical data and isolates from 95 consecutive cases of keratitis associated with *S. aureus* were collected by the MOG over a 2 year period, as described in chapter 2.11. Isolates were sent to the microbiology laboratory at the Royal Liverpool University Hospital NHS Trust where they were sub-cultured and stored on beads. MICs of ciprofloxacin, moxifloxacin, levofloxacin, penicillin, vancomycin, teicoplanin, chloramphenicol, gentamicin, meropenem, linezolid and tigecycline were determined using E-Tests (as described in chapter 2.21).

3.32 Detection of mecA and lukSF-PV

DNA was extracted from the *S. aureus* isolates collected, using QIASymphony SP (chapter 2.21). A multiplex PCR assay was performed using 50-70 $ngml^{-1}$of the extracted DNA. Primers used were as follows: (1) Staph756F and Staph750R; amplifying the *Staphylococcus* genus-specific 16S rRNA gene (positive internal control), (2) Luk-PV-1 and Luk-PV-2; amplifying the *lukSF-PV* gene and (3) mecA1 and mecA2; amplifying the *mecA* gene, a determinant of MRSA. See table 6a for primer details. The optimized multiplex PCR conditions were as described by McLure et al.[178] Thermocycling conditions were; 94 °C for 10 minutes, 10 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 75 s and 25 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 75 s. Amplification was assessed by running PCR products on 1% agarose gels stained with ethidium bromide. Positive controls were USA300 (positive for 16S, *mecA* and *lukSF-PV*) and SH1000 (positive for 16S).

3.33 lukSF-PV sequencing

Isolates that were PCR positive for *lukSF-PV* underwent further genetic analysis. PCR amplification of an internal fragment (+108 to +1807) of the *lukSF-PV* operon was performed. The 25 µl PCR mixture contained 50-70 ngml^{-1}of bacterial DNA, 1.25 units Accuzyme, 12.5 µl Biomix red (Bioline), 2.5 mM magnesium chloride and 100 pmol primers; PVL1F, PVL1R, PVLint2F, PVLint2R, PVLint3F and PVLint3R (table 6b for primer details). PCR cycle conditions were as follows: 5 minutes at 95 ^{o}C, followed by 30 cycles of 1 minute at 94 ^{o}C, 1 minute at 50 ^{o}C and 2 minutes at 72 ^{o}C. PCR products were then sequenced (GATC Biotech, Germany) and single nucleotide sequences were built for each isolate utilising a multiple sequence alignment tool from Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).[179]

3.34 Multi Locus Sequence Typing

Background: Multilocus sequence typing (MLST) is a method of characterising bacterial isolates on the basis of the sequences of seven housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*.[180] For each gene fragment the sequences are assigned as distinct alleles, and each isolate is defined by these alleles known as a sequence type (ST). Isolates with the same allelic profile can be assigned as members of the same clone allowing comparisons with other studies. Appendix C lists the full amino acid sequence for each gene.

Methods: MLST was performed with DNA extracted from isolates from the MOG collection. PCR amplicons of the 7 *S. aureus* housekeeping genes were sequenced (primer details table 6c) and the loci of each gene was constructed from reads using the Clustal Omega tool described in section 3.33. STs were determined using the database provided by http://www.MLST.net.

3.35 Clinical outcome data collection

Clinical outcome data was collected in a similar manner to a previously validated method by Kaye et al.[181] Parameters collected were; ulcer size (mm^2) at presentation, scar size (mm^2) at final examination, healing time (days) defined as interval to epithelialisation, treatment time (days), risk factors for infection, surgical interventions (amniotic membrane grafts, penetrating keratoplasty or evisceration/enucleation), and the ratios of ulcer size to healing time and ulcer size to treatment time. For each parameter, patients were assigned a positive or negative clinical outcome score according to the deviation of the parameter in standard deviations (SD) from the mean. For example, if the ulcer size was within 1 SD of mean it was scored 0, between 1 and 2 SD they scored 1, and >2 SD they scored 2. Similarly, negative scores were given for negative SD values. Surgical events were assigned a score of 3 and loss of the eye a score of 5. Aggregate clinical scores were then calculated for all parameters.

3.36 Statistical Analysis

Logarithms were taken for all clinical and MIC data, except for discrete events such as surgical intervention or loss of the eye. The Kruskal-Wallis test (SPSS version 20) was used to determine differences between groups.

3.4 Results

3.41 LukSF-PV presence and type

Each individual PCR yielded fragments of the expected sizes, i.e., 756, 433, and 310 bp for the 16S rRNA, *lukS/F-PV*, and *mecA* genes, respectively. Of the 95 *S. aureus* keratitis isolates; 9 (9.5%) were *lukSF-PV*+ve, 9 (9.5%) were *mecA*+ve

and 1 isolate was positive for both (1.1%) (figure 13). The *lukSF-PV* gene was highly conserved in 8 isolates. One amplification was unsuccessful despite numerous attempts. There was, however, nucleotide variation in seven of the isolates compared to the USA300 reference strain (figure 14). In total there were 5 nucleotide substitutions causing 4 amino acid changes. Two major sequence variants of *lukSF-PV*, the R and H variants, were identified, based on a substitution of adenine for guanine at nucleotide 528 that resulted in a histidine (H) to arginine (R) amino acid change. MLST typing showed that *lukSF-PV*+ve isolates could be classified into two diverse STs: 3 of the 9 were classified as ST30 and the others were STs 12, 49, 1, 121 and 8. A summary of the gene sequencing and MLST results is included in table 9.

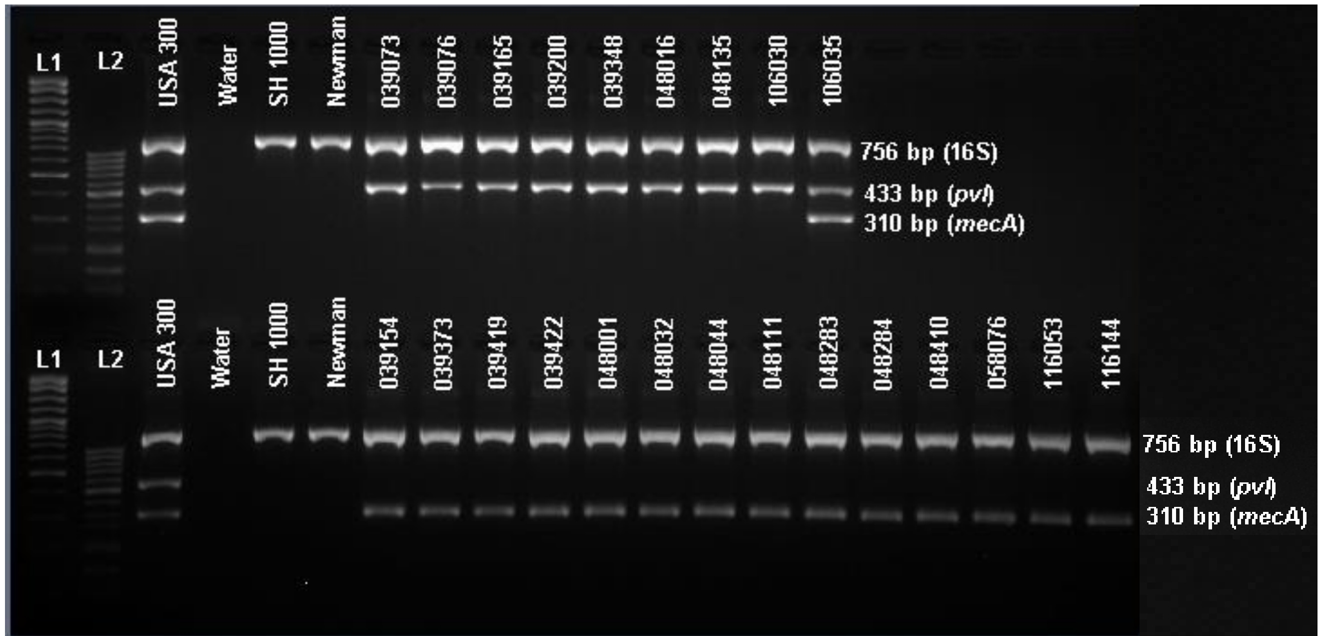


Figure 13. PCR of *S. aureus* isolates positive for *pvl* and *mecA*. Positive controls USA 300 (positive for *lukSF*-PV and *mecA*), SH1000 and Newman (positive for 16s).

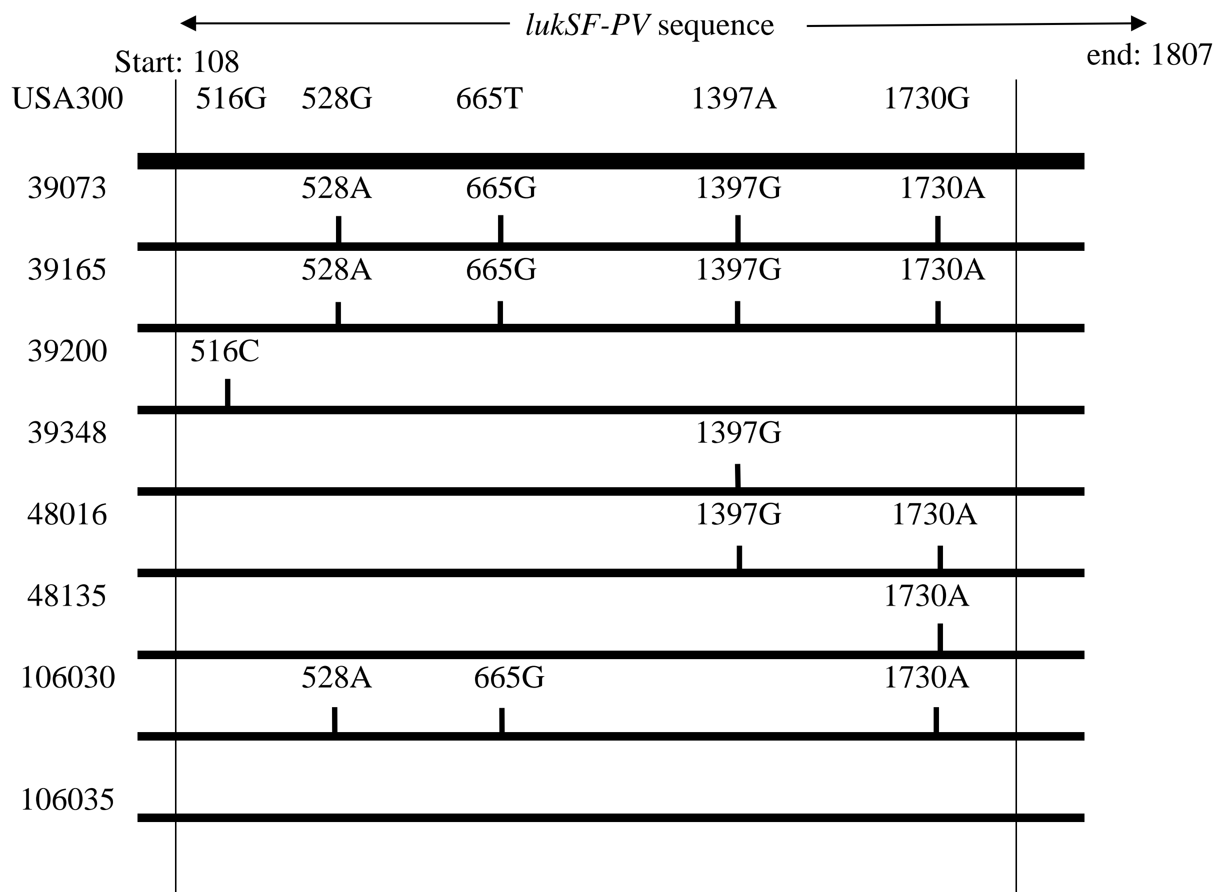


Figure 14: Schematic representation of sequence variance between the 8 *lukSF-PV*+ve *S. aureus* isolates sequenced. Strain identity numbers are indicated on the left with the USA300 used as a comparator reference strain at the top. The position of amino acid substitutions in relation to the USA300 strain are indicated for each strain followed by the alternative base. For example, for strain 39200; at position 516 amino acid G in USA300 was replaced with C. A = Adenine, T = Thymine, C = Cytosine, G = Guanine

Table 9. Gene sequencing and Multi Locus Sequence Typing (MLST) of *lukSF-PV*+ve *S. aureus* isolates

Isolate No.	Location	*mecA*	Genotype variant	MLST type
39073	London	-ve	R	30
39076	London	-ve	N/A	12
39165	London	-ve	H	49
39200	Manchester	-ve	H	1
39348	Birmingham	-ve	H	121
48016	London	-ve	H	30
48135	London	-ve	H	30
106030	London	-ve	R	1
106035	London	+ve	H	8

3.42 Antimicrobial sensitivity patterns

The MICs of the 95 isolates to 11 antimicrobials tested are shown in table 10. There was no significant difference in the mean MIC between the *lukSF-PV/mecA*-ve and *lukSF-PV*+ve groups, apart for tigecycline which had a significantly lower mean MIC for the *lukSF-PV*+ve compared to both the *mecA*+ve and *lukSF-PV/mecA*-ve isolates. There was greater variation in the MICs of the *lukSF-PV* +ve isolates than that of the *lukSF-PV/mecA*-ve isolates to the fluoroquinolones and penicillin as evident in the higher MIC_{90} (antimicrobial concentration required to inhibit the growth of 90% of organisms). In contrast, the *mecA*+ve isolates had significantly higher mean MICs ($p<0.05$) with higher MIC_{90} and MIC_{50} to meropenem and the fluoroquinolones.

3.43 Clinical outcome

Ninety of the patients had received a fluoroquinolone (ofloxacin or ciprofloxacin) as initial treatment. Five of the patients with isolates negative for both *lukSF-PV*

and *mecA* had received chloramphenicol as initial treatment. There were no significant differences in age, gender distribution or position of the ulcer (distance from the limbus) between the groups that were positive or negative for *lukSF-PV* or *mecA* (p= 0.40). The healing and treatment times, ulcer and scar size and overall clinical score tended to be greater in the *lukSF-PV*+ve group (Table 11). The proportion of patients that required surgery as management for their keratitis was significantly greater in the *lukSF-PV*+ve group (3 of 9 cases required surgery) compared to the *lukSF-PV*-ve group (3 of 81 cases required surgery) or the *mecA*+ve group (1 of 9 required surgery) (p=0.016). In comparison to patients with *lukSF-PV*-ve isolates, the odds ratio for patients with *lukSF-PV*+ve isolates requiring surgery was 7.8 (95% CI 1-42, p=0.018) whilst those with *mecA* isolates was 2.6 (95% CI 0.3-27, p=0.42).

Table 10. Antimicrobial susceptibility data for patients with *S. aureus* keratitis who were *lukSF-PV*+ve (n=9), *mecA*+ve (n=9) or negative for both (*lukSF-PV/mecA* -ve (n=77). Mean MIC and the antimicrobial concentration at which 90% and 50% of bacteria are inhibited (MIC_{90} and MIC_{90} respectively). Antimicrobials tested were ciprofloxacin (Cip), moxifloxacin (Mox), levofloxacin (Lev), penicillin (Pen), teicoplanin (Tei), chloramphenicol (Chl), gentamicin (Gen), meropenem (Mer), linezolid (Lnz) and tigecycline (Tig).

		Cip	Mox	Lev	Pen	Tei	Chl	Gen	Mer	Lnz	Tig
lukSF-PV-ve	Mean MIC	0.5	0.03	0.1	0.4	0.9	3.6	0.4	0.03	0.4	0.1
mecA-ve	MIC_{50}	0.4	0.03	0.1	0.8	1.0	3.0	0.4	0.02	0.5	0.1
	MIC_{90}	1.5	0.05	0.2	1.5	1.5	8.0	1.0	0.05	0.8	0.1
lukSF-PV+ve	Mean MIC	0.8	0.1	0.2	0.3	0.6	2.4	0.4	0.03	0.4	0.04*
	MIC_{50}	0.4	0.1	0.1	0.5	0.8	2.0	0.4	0.03	0.5	0.1
	MIC_{90}	32	1.6	25.7	7.2	1.0	4.2	0.6	0.1	0.6	0.1
mecA+ve	Mean MIC	20*	2*	12*	12*	0.4*	6.2	0.3	0.7*	0.4	0.1
	MIC_{50}	32	1.0	8.0	32.0	0.3	3.5	0.4	0.9	0.4	0.1
	MIC_{90}	32	4.0	38.0	32.0	1.6	131	0.5	2.3	0.6	0.1

*significant difference between the mean MIC of the three *S. aureus* groups. The mean MIC for teicoplanin was significantly lower in the *lukSF-PV*+ve group of isolates.

Table 11. Clinical outcome (mean and SD) for patients with *S. aureus* keratitis; *lukSF-PV*+ve (n=6), *mecA*+ve (n=8) or negative for both (*lukSF-PV/mecA* -ve (n=70). Parameters analysed: Treatment time (TT), healing time (HT), ulcer area (UA), scar area (SA), ulcer to scar area (UA/SA), healing time to ulcer area (HT/UA) and clinical outcome score (CS) according to the deviation of the parameter in standard deviations (SD) from the mean and % of patients requiring surgery (Sur).

		TT (days)	HT (days)	UA (mm^2)	SA (mm^2)	UA/ SA	HT/ UA	CS	Sur (%)
lukSF-PV-ve and *mecA* –ve	Mean	20.2	9.7	3.1	1.6	0.8	3.4	2.1	3.7
	SD	26.3	12.6	11.8	7.6	1.1	5.6	2.8	
lukSF-PV +ve	Mean	20.6	13.6	5.3	3.4	0.7	2.4	3.6	33
	SD	31.7	27.0	5.9	4.3	0.4	2.8	3.7	
mecA +ve	Mean	15.5	6.2	2.2	1.0	0.7	3.4	1.3	11
	SD	20.3	3.4	3.7	4.2	0.3	5.3	1.9	

3.5 Discussion

Interest in PVL toxin as a *S. aureus* virulence factor intensified after it was associated with fatal cases of community acquired pneumonia in previously healthy children.[151] Yoong et al[160] identified that PVL induces pro-inflammatory cytokines and a moderate TNF-α response in pulmonary infections in a murine model, indicating a possible immunomodulatory role. The same group had previously found that mutant PVL strains with isogenic *lukS* and *lukF* deletions were more virulent in a murine skin abscess model compared to wild type.[161] Further studies using murine models of sepsis, abscess, skin infection and pneumonia showed similar results.[162, 163] As previously mentioned there has been much discussion about the suitability of using mice to study PVL toxicity, due to

the differences in mouse and human immunology, in particular, the insensitivity of mouse neutrophils to the effects of the leukocidin.[166] There is a growing consensus that a rabbit model might yield more meaningful results. *In vivo* studies in rabbits indicate that the presence of PVL increases the virulence of *S. aureus* strains.[164, 165] Diep et al[165] identified a role for PVL in the virulence of *S. aureus* USA300 and USA400 strains during the early, acute stages of bacteraemia in rabbits, when lysis of PMNs might allow colonisation to be established. These findings were supported by Lipinska et al,[166] who noted that during the early course of skin infection in a rabbit model a PVL+ve USA300 isolate of *S. aureus* produced larger lesions and more skin necrosis, compared to PVL knockout strains. There is also debate on the role of PVL in *S. aureus* infection in patients. There are several studies which suggest that there is no difference in clinical outcome with PVL+ve MRSA infections or that the presence of PVL can reduce virulence.[160, 167-169] The presence, however, of PVL in the virulent CA-MRSA clone USA300[182] lends epidemiological support that the toxin influences the virulence of *S. aureus*. Indeed a correlation between PVL carriage and invasiveness of disease, as well as virulence, has been found not only in USA300, but also in other community-acquired strains.[170-172] Although these hyper-virulent lineages are known to be MRSA, attention must also be paid to PVL+ve meticillin-sensitive *S. aureus* (MSSA), which represents the vast majority of UK PVL-containing clones.[174]

In this study of 95 patients with *S. aureus* keratitis, 9.5% of isolates were *lukSF-PV*+ve and these cases tended to have larger ulcers and worse outcomes, in particular a significantly higher incidence of cases requiring surgical intervention. These findings reflect those of Muttaiyah et al[173] who found a statistically

significant correlation between PVL+ve MSSA and the need for surgical intervention, amongst 411 isolates taken from patients with a variety of *S. aureus* infections. The data does not suggest a link between a particular allele of *lukSF-PV* and keratitis, as 6 different *lukSF-PV* alleles were identified, including both H and R variants. The predominant subgroup was the H variant, which is consistent with the findings of Wolter et al[183] who identified the H variant as the most common form in *S. aureus* isolates from Europe.

It is noteworthy, that 8 of the 9 *lukSF-PV*+ve isolates in this study were *mecA*-ve. The MRSA status of a *S. aureus* isolate is determined by the *mecA* gene that lies in the *SCCmec* cassette (chapter 5). It has been proposed that the presence of *mecA* may impose a fitness cost on isolates in environments such as the community with limited antimicrobial use. This may result in evolutionary pressure for some CA-MRSA strains to lose the SCC*mec* cassette while retaining key virulence or fitness factors such as *lukSF-PV*.[184] Alternatively, the MSSA strains may have never acquired the *SCCmec* cassette. The small number of *lukSF-PV*+ve isolates in this study makes it difficult to speculate why only 1 of 9 *lukSF-PV*+ve was *mecA*+ve.

The diversity in the sequence types observed in this study is in agreement with the clonal diversity observed in MSSA isolated from skin and soft tissue infections in the USA.[184] The presence of multiple PVL alleles within the same genetic lineage, for example the presence of both H variants and R variants as well as 3 different single nuclear polymorphisms (SNPs) among the ST30 isolates, could indicate horizontal gene transfer of the PVL phage between different clonal complexes.

Multiplex PCR as described by McClure et al[178] is a relatively quick and inexpensive way to screen for both *lukSF-PV* and *mecA* using the 16S rRNA gene locus as a positive control. The higher MICs seen to the fluoroquinolones for *mecA* isolates reflects that reported in previous studies on patients with keratitis.[104, 185] In contrast, the mean MICs for *lukSF-PV* and *lukSFPV*-ve groups were similar for the antimicrobials tested. Of note however, was the lower mean MIC of *lukSF-PV* to tigecycline compared to both the *lukSF-PV*-ve and *mecA*+ve *S. aureus* isolates.

3.6 Conclusion

The study found that patients with *lukSF-PV*+ve *S. aureus* were associated with a trend to worse clinical outcome and more surgical interventions, with an effect unrelated to MICs. This suggests that *lukSF-PV* may be an important virulence factor in *S. aureus* associated keratitis. A larger study would be needed to verify this, especially in light of differing effects on clinical outcome that PVL has been shown to have, in previous studies.[167, 168, 173, 186]

CHAPTER 4 *PSEUDOMONAS AERUGINOSA*

4.1 Introduction

P. aeruginosa is an important Gram-negative human pathogen. It is a frequent cause of hospital acquired infections such as pneumonia, urinary tract infections and bacteraemia. *P. aeruginosa* is an opportunistic pathogen, only rarely causing disease in healthy people. In most cases of infection, the integrity of a physical barrier to infection (e.g. cornea, skin) is lost, or an underlying immune deficiency[187] (e.g. neutropenia, immunosuppression) is present. Patients with cystic fibrosis are at an increased of infection due to *P. aeruginosa* colonisation in excess secretions.[188] Adding to its pathogenicity, this bacterium has minimal nutritional requirements and can tolerate a wide variety of physical conditions. *P. aeruginosa* is one of the most frequent causative bacteria implicated in keratitis in most studies (table 3). It constituted 20.9 % of isolates collected from patients with keratitis in the UK by the MOG.

4.11 Clinical features

Contact lens use significantly increases the susceptibility of the cornea to *P. aeruginosa*[19,136] (chapter 1.1311). A detailed contact lens history, including type of lens, extent of wear and lens hygiene, should therefore be taken from all patients presenting with keratitis (see chapter 1.121).

P. aeruginosa associated keratitis is typically rapid in onset.[121] Early signs of infection include a grey epithelial and stromal microinfiltration with oedema. The stromal infiltration may extend horizontally and vertically involving the whole width and depth of the cornea and a yellow-green mucopurulent discharge

is typically seen. A severe anterior chamber reaction is often seen, sometimes with the development of a hypopyon. During the next 2-3 days, if untreated, a ring infiltration may develop, with scleral and corneal melting. If the ulcer progresses, descemetocele formation may occur with subsequent corneal perforation.[189] Figure 1 shows an example of keratitis caused by *P. aeruginosa.*

4.12 Virulence factors

The large array of bacterial surface factors and active cellular processes that contribute to the virulence of *P. aeruginosa* will now be described (summarised in table 12).

Table 12. Summary of virulence factors associated with *P. aeruginosa*

Pathogenic function	Virulence factor
Defence against bactericidal reaction	
Complement inhibition	Proteases, alkaline protease, protease IV, alginate *Pseudomonas aeruginosa* small protease, large exoprotease
Host invasion/cytotoxicity	
Adhesion	Type IV pili, lectins
Invasion	Glycocalix, biofilms, elastases (Las B and Las A), Alkaline protease, haemolysins (phospholipases and rhamnolipid), cytotoxin (leukocidin) pyocyanin pigment, siderophores and siderophore uptake systems, endotoxin, LecA and LecB lectins Type III secretion system: PcrV, PopB, and PopD and *exoS, exoT, exoU*, *exoY*
Inter-bacterial communication	Quorum sensing: *las* and *rhl* systems *Pseudomonas* quinolone system
Motility/chemotaxis	Flagella, retractile pili

4.121 Bacterial survival

P. aeruginosa has developed a number of strategies to combat the vast repertoire of host defences in humans.[190]

P. aeruginosa can evade detection by the host by directly destroying host immune molecules that are involved in pathogen detection. For example, an important component of host defence is the deposition of a complement component C3b on the bacterial surface, leading to pathogen destruction. *P. aeruginosa* is able to counter complement activation by producing alginate to limit accessibility of complement, and proteases (including alkaline protease and elastase) that degrade C3b.[191]

P. aeruginosa can also down-regulate their own expression of PAMPs (chapter 1.332) in order to "hide" from the host immune system. The flagellum, a virulence determinant required for motility and attachment, is also a PAMP that is detected by the host. It has been shown that upon growth on purulent mucus, *P. aeruginosa* down-regulates flagellin synthesis, thereby dampening the host immune response.[192]

Reactive oxygen species produced by neutrophils are neutralized by the deployment of the antioxidant enzymes known as catalases. *P. aeruginosa* produces a catalase that is not dependent on the presence of iron, known as nonhaem catalase KatN (chapter 4.133).[193]

4.122 Bacterial adhesion and invasion

4.1221 Twitching motility

P. aeruginosa spread rapidly across surfaces by twitching motility as a result of the repeated extension, tethering, and retraction of long surface filamentous appendages, called type IV pili.[194] Twitching has been implicated in the virulence of *P. aeruginosa* associated disease including pneumonia and keratitis.[195] Zolfaghar et al[196] found twitching motility mutants of *P. aeruginosa* had reduced virulence in a murine keratitis model, compared to twitching motility-competent wild-type bacteria. It is postulated that type IV pili have a role in epithelial adherence and traversal,[87] as well being one of the many factors in biofilm formation (chapter 1.33).

4.1222 Type III secretion system

Of the many bacterial factors that contribute to the pathogenesis of *P. aeruginosa*, the type III secretion system (TTSS) is important for bacterial persistence in the presence of host defence mechanisms and has been associated with poor clinical outcomes.[197] The TTSS consists of 43 genes that encode the secretion apparatus, a translocon and factors that regulate secretion.[198] The secretion apparatus exports toxins from across the bacterial cell envelope, and the translocon assembled by the proteins PcrV, PopB, and PopD, is responsible for injecting these toxins into the host cell.[199] Four secreted toxins, or exotoxins, have been identified to date: *exoU*, *exoS, exoT*, and *exoY*. The first two have been closely linked to virulence in keratitis and will be discussed here.

The contribution of *exoU* to virulence is attributable to its phospholipase A2 activity.[200-202] Upon injection into host cells, *exoU* is activated and targeted to

the plasma membrane, where it cleaves membrane phospholipids, resulting in rapid and complete cell lysis. *ExoS* has invasive properties and encodes both GTPase-activating protein and ADP-ribosyltransferase activities.[203] It induces rapid lysis of macrophages, epithelial cells and fibroblasts.

Most *P. aeruginosa* strains contain either *exoS* or *exoU.* Strains containing both genes, however, are uncommon.[204] Isolates from keratitis have been found to be disproportionately carriers of *exoU* (rather than *exoS*) in comparison with the wider *P. aeruginosa* population.[205] *exoU*-positive strains are associated with greater morbidity in *P. aeruginosa* keratitis.[206, 207 208]

4.1223 Proteases

Proteases are enzymes that hydrolyse peptide bonds in proteins and contribute to the corneal melting that occurs in severe cases of keratitis. *P. aeruginosa* is capable of secreting at least seven different proteases[142]; elastase A (Las A), elastase B (Las B), modified elastase, alkaline protease, protease IV, *Pseudomonas aeruginosa* small protease, and the large exoprotease. Las A, Las B, modified elastase, and AP are metalloproteinases and may be produced by only some strains.

4.1224 Quorum sensing-regulated factors

QS-regulated factors (see chapter 1.33) have an essential role in *P. aeruginosa* virulence. They regulate around 350 genes (6% of the *P. aeruginosa* genome), playing critical roles in biofilm formation[101, 209] and production of numerous toxins.[210] The commonest QS molecules in *P. aeruginosa*, (as well as most Gram-negative bacteria) are acyl homoserine lactones (AHLs). When the concentration

of AHLs in the intracellular environment increases (due to increasing numbers of bacteria) transcriptional regulators are induced. Three QS systems have been extensively studied in *P. aeruginosa*; *las, rhl* and *Pseudomonas* Quinolone Signal (PQS).[211] The *las* system consists of LasR, the regulator protein and LasI synthase protein, which is essential for the production of the AHL signal molecule N-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL). The *rhl* system consists of RhlI and RhlR proteins. The RhlI synthase produces the AHL N-butyryl-L-homoserine lactone (C_4-HSL), and RhlR is the transcriptional regulator. PQS has only recently been identified in *P. aeruginosa.* Häussler[212] postulated that PQS is an essential mediator of formation of the population structure of *Pseudomonas* and its survival in hostile environmental conditions. On the one hand it acts as a pro-oxidant and sensitizes the bacteria towards oxidative and other stresses and, on the other, it induces a protective anti-oxidative stress response. This dual function may be beneficial to *Pseudomonas* populations in promoting survival of the fittest, and in contributing to bacterial multi-cellular behaviour. McKnight et al[213] suggest that PQS acts as a link between the *las* and *rhl* QS systems and that this signal is not involved in sensing cell density.

4.123 Invasive and cytotoxic *P. aeruginosa*

Two important virulence determinants in *P. aeruginosa* are invasiveness and cytotoxicity which are due in part to the possession of two mutually exclusive effector exotoxins of the type III secretion system; *exoU* or *exoS* (chapter 4.1222).[207] Invasive *P. aeruginosa* strains encode *exoS* and can sequester themselves intracellularly, replicating and stimulating membrane bleb formation within host cells. Cytotoxic strains lack *exoS* and instead encode the cytotoxin *exoU*, which can quickly kill cells without being sequestered inside the host cell.

Borkar et al[214] showed that bacterial keratitis caused by invasive (*exoS*+ve) strains was associated at presentation with significantly better visual acuity than cytotoxic (*exoU*+ve) strains, but had less improvement in visual acuity at 3 months. They also revealed that adjunctive treatment with topical steroids had a different effect on cytotoxic and invasive strains. This illustrates the concept that not all infections caused by pathogens of a single species present or respond to treatment similarly.

4.124 *P. aeruginosa* reference strains

PAO1 and PA14 are two frequently used reference strains used in *P. aeruginosa* studies. The PAO1 strain (originally called *P. aeruginosa* strain 1) was first isolated from a patient's wound over 50 years ago in Australia.[215] It became the first reference strain for Pseudomonas genetics and analyses of the physiology and metabolism of this bacterium. With the advent of pulsed-field gel electrophoresis (PFGE), a physical map of the PAO1 genome was constructed and later merged with the genetic map information.[216] By 2000 the PAO1 strain had been completely sequenced.[217] PAO1 is known to encode *exoS* and is therefore classified as an invasive *P. aeruginosa* strain.[206] PAO1 is used as the reference strain in in Array Tube genotyping (chapter 4.131).

PA14 (originally called UCBPP-PA14) is a clinical isolate taken from a human burn patient. The genome of PA14 was published in 2004 and revealed a high degree of conservation compared to PAO1.[218] PA14 has been shown to be much more virulent than PAO1 in a number of diverse models of infection, leading to the hypothesis that PA14 is a multihost pathogen capable of infecting invertebrate and vertebrate animal species and plant species.[219] The virulence of PA14 has been attributed to two pathogenicity islands; PAPI-1 and PAPI-2, that

are absent in PAO1.[220] PAPI-1 encodes a number of likely virulence factors, including type IVB pili[221] and PvrR, a regulator involved in biofilm synthesis.[222] PAPI-2 encodes the cytotoxin *exoU* of the TTSS and is therefore classified as a cytotoxic strain.[223,224]

4.13 Genotypic features of P. aeruginosa clones

A range of genotyping methods have been used to study *P. aeruginosa* isolates, including pulse field gel electrophoresis,[225] Random Amplification of Polymorphic DNA,[226] MLST,[225, 227] and more recently the Clondiag Array Tube (AT).[228]

4.131 Array Tube genotyping

The AT was developed in 2007 and consists of 77 oligonucleotides immobilised into a microchip and embedded into the base of a tube.[229] The layout is divided into four sections, as shown in Figure 15. The chip has 29 markers for a range of genomic islands and islets, (boxed in red), 15 markers for variable genes, (boxed in blue), a LES PS21 marker, (boxed in black) and 16 markers relating to single nuclear polymorphism (SNP) loci, (boxed in green). The markers for genomic islands, variable genes and the LES PS21 marker are represented by two spots and the SNPs by four spots. There are six control spots (four in the first column and two in the last) that indicate the efficiency of hybridisation, conjugation and precipitation.

Interpretation of the AT chip

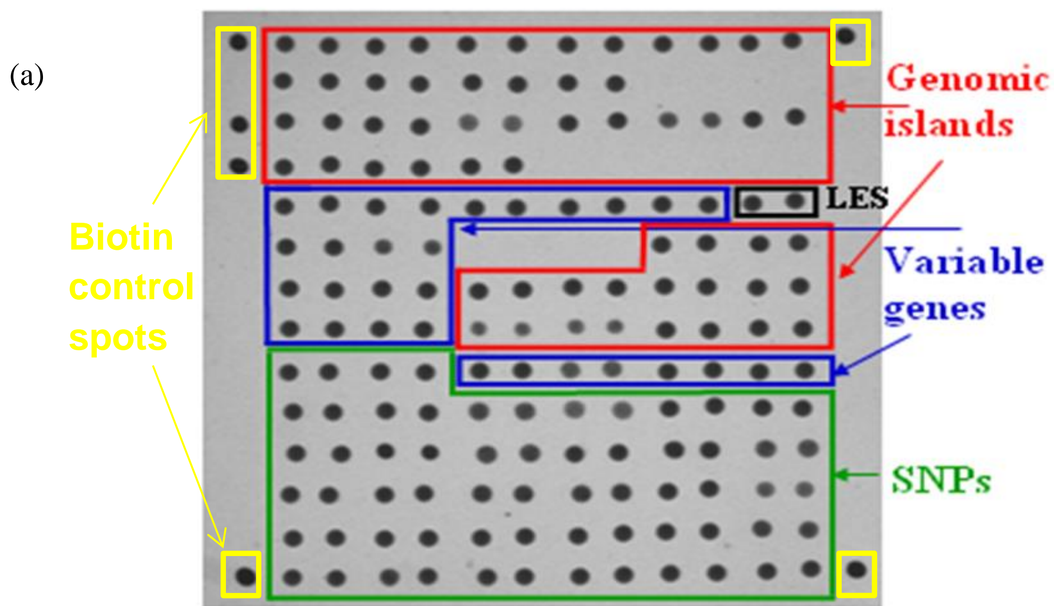
A genetic profile can be determined by analysis of thirteen SNP patterns at seven conserved loci ampC, citS, alkB2, fliCa, oprI, oprL and oriC and the presence or absence of 3 variable genes encoding the type III secretion virulence proteins

(exoU and exoS) and the flagellin protein FliC. These patterns for each locus are compared to a predetermined table (Figure 16). The genomic islands (red) and variable genes (blue) are represented by two oligonucleotides and the presence of the spots indicates whether the target is present or absent. If the gene is present the strain is assigned a '1' for that locus and '0' if the gene is absent. In contrast each SNP is represented by four oligonucleotides, two on the left that have sequences that match with PAO1, and two on the right, with sequences that don't match PAO1 (considered a "mutant strain"). If the hybridisation spots are stronger on the left (the PAO1 sequence) the strain is assigned a '0' for that locus and a '1' if the hybridisation is stronger for the spots on the right.

The resulting hexadecimal code can then be shortened to a 4 digit code as shown in Figure 17 and an individual genetic fingerprint is produced. This code can be compared to a large, previously described database[229] and the strain can be assigned a clone type.

The AT system has been used to successfully genotype *P. aeruginosa* isolates from a range of chronic and acute clinical conditions, including chronic obstructive pulmonary disease[230] and more recently by Stewart et al[231] on a selection of keratitis associated strains.

(a)



(b)

C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1
PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6		
pKL-1	pKL-3	TB-C47-1	TB-C47-2	PAPI-1-Pili ch	PAPI-1-LuBiPr.
pKLC-unknown	pKLC-adhesion	pKLC-metabolism			
Pyov.Rec.I	Pyov.Rec.IIa	Pyov.Rec.IIb	Pyov.Rec.III	Pyov.Rec.B	LES
PA0636	PA0722			PAGI-1	PA0980
PA0728	PA2185	Fla-island-1	Fla-2-orfA	47D7-1	PAPI-2-Actr
PA2221	PA3835	Fla-2orfI	Fla-2orfj	47D7-2	PAPI-2-xF1753
ampC-7		fliCa	fliCb	exoS	exoU
ampC-4		ampC-5		ampC-6	
oprI		ampC-1		ampC-3	
citS-1		citS-2		oprI	
fliCa		fliCa		alkB2	
oriC		oprL a		oprL b	

Figure 15. Sequence positions on Array Tube. Genomic islands and islets, (boxed in red), variable genes, (boxed in blue), LES PS21 marker, (boxed in black) and 16 markers relating to single nuclear polymorphism (SNPs) loci, (boxed in green). Markers for genomic islands, variable genes and the LES PS21 marker are represented by two spots and the SNPs by four spots. Six biotin control spots (boxed in yellow). SNPs and variable markers were used for strain identification.

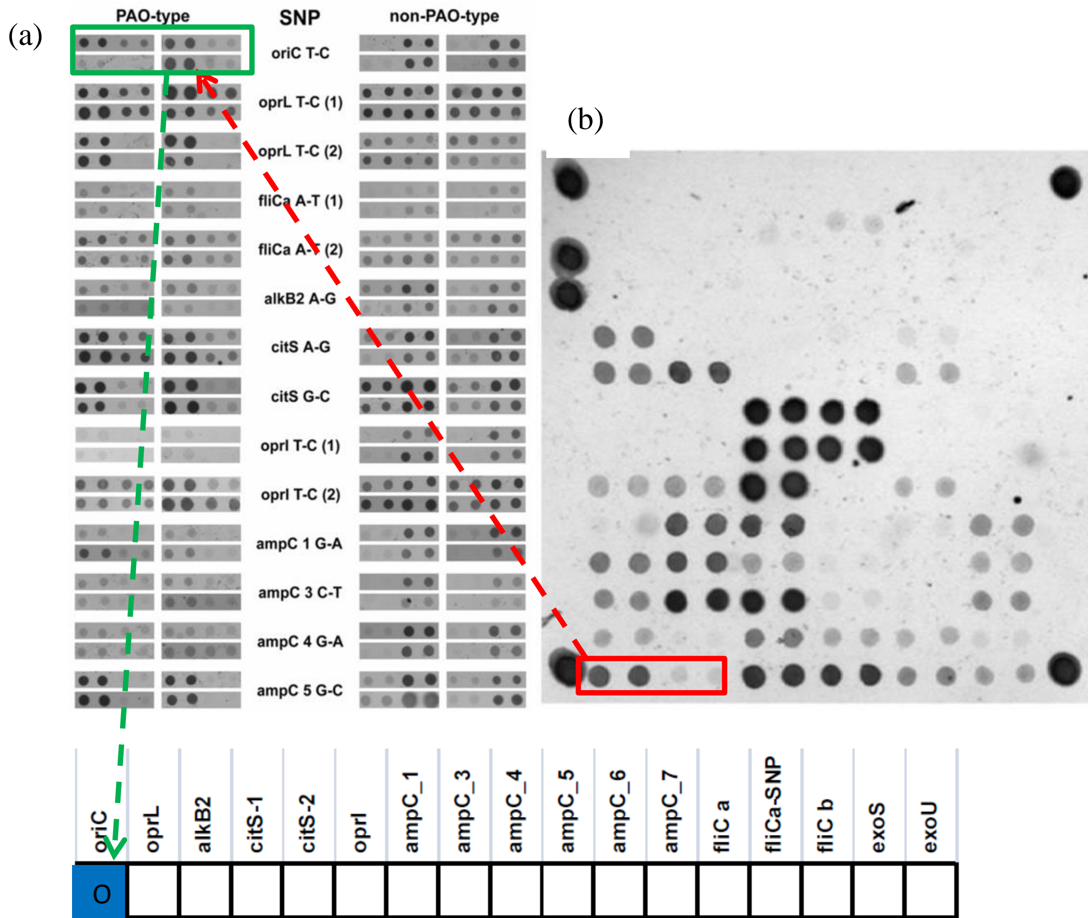


Figure 16. Method of generating hexadecimal code from Array Tube image. (a) predetermined table for comparison of precipitation patterns for the seven conserved loci, (b) example of Array Tube image generated from *P. aeruginosa* isolate. In this example oriC SNP (boxed in red) compared to oriC on table (boxed in green). As oriC pattern in the isolate is comparable to the PAO1 strain it is annotated as “0” in the hexadecimal code. In this way the entire hexadecimal code is generated.

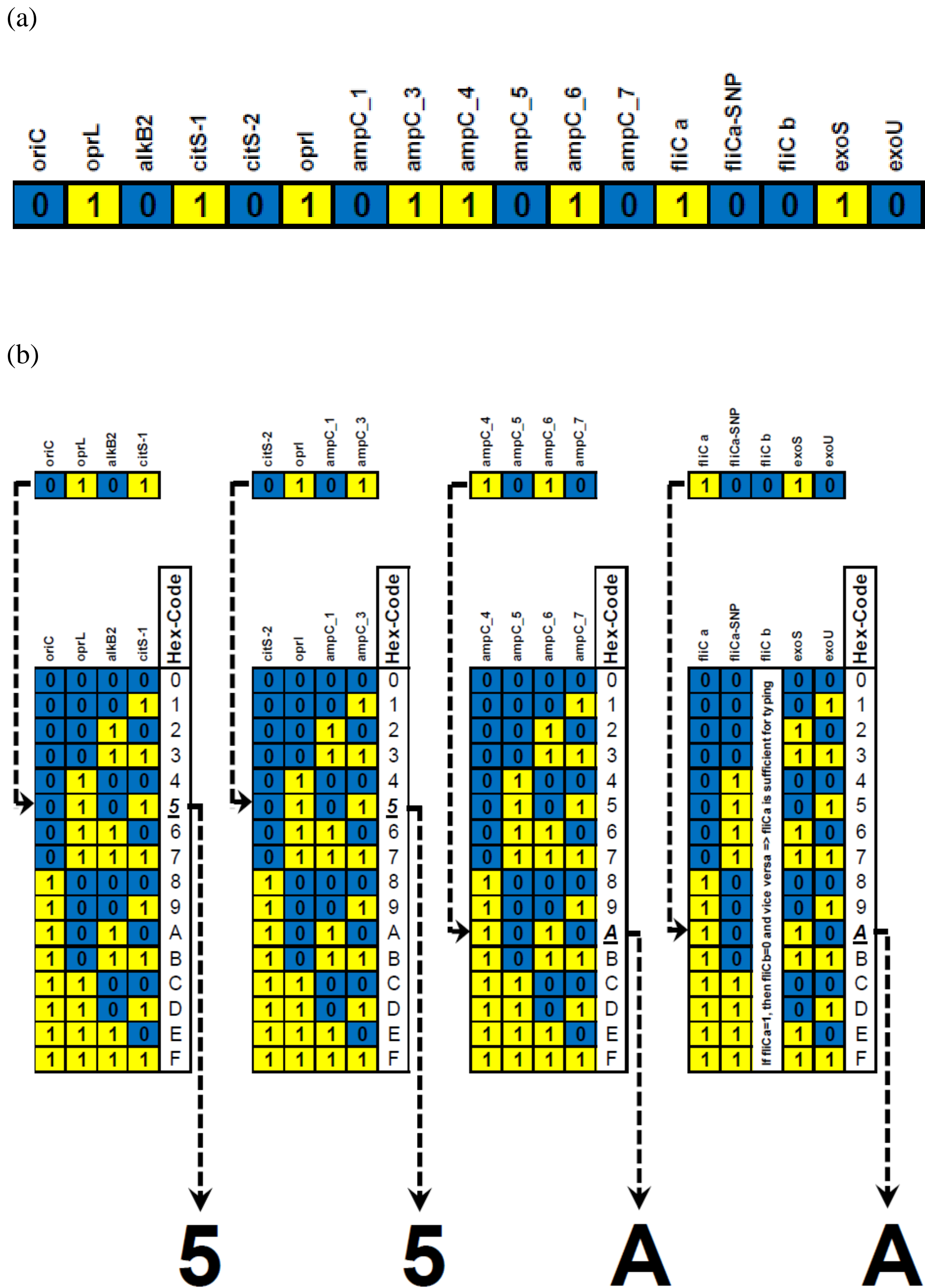


Figure 17. Conversion of Array Tube hexadecimal code into four digit code. (a) hexadecimal code generated from Array Tube image from Figure 16. (b) hexadecimal code separated it into four parts. Four corresponding code digits are found by comparing to a predetermined table. Originally described by Wiehlmann et al.[229]

4.132 Population genotyping

Population genotyping is the method of displaying the genetic relationships between isolates of a bacterial population. The BURST algorithm (which stands for; Based Upon Related Sequence Types) is a web-implemented clustering algorithm designed for use on genotype data sets from bacteria. BURST was devised and developed by Ed Feil from the University of Bath.[232] An enhanced version of BURST (eBURST version 1) was developed and integrated into the website www.mlst.net.[233] The latest version; eBURST version 3, has been developed with funding from the Wellcome Trust by and contains several new features improving on previous versions.

eBURST uses a simple model of bacterial evolution based on a founding or ancestral genotype that increases within a population and, in doing so, begins to diversify to produce a cluster of closely related genotypes. It then predicts the descent from the founder genotype and displays the output as a radial diagram (Figure 18). Although the eBURST method has been applied mostly to MLST data, it can also be used to display relationships between strains using other data, such as the AT genotyping data.

The first step of the process is to divide the input data into groups of STs that have some level of similarity in their allelic profiles. Within a group, all the STs must be a single locus variant (SLV) of at least one other ST in the group. The primary founder of the group is defined as the ST that differs from the largest number of other STs at only a single locus. If two STs have the same number of associated SLVs, the one with the largest number of double-locus variants (DLVs) is selected as the founding ST. A clonal complex is a set of STs that are believed to be descended from the same founding genotype. More than one cluster of

linked STs (clonal complex) may be displayed in the eBURST diagram, along with a number of unlinked individual STs or 'outliers'.

The eBURST diagram displays the patterns of descent within each group from the predicted founder ST in a radial fashion, with lines showing the links from the founder to each of its SLVs. The circle representing the predicted founder is coloured blue. In a large eBURST group there may be several STs that have a number of SLVs of their own. A ST that has diversified to produce multiple SLVs is called a subgroup founder and these are depicted by a yellow circle. The size of the circle for each ST represents its abundance within the population.

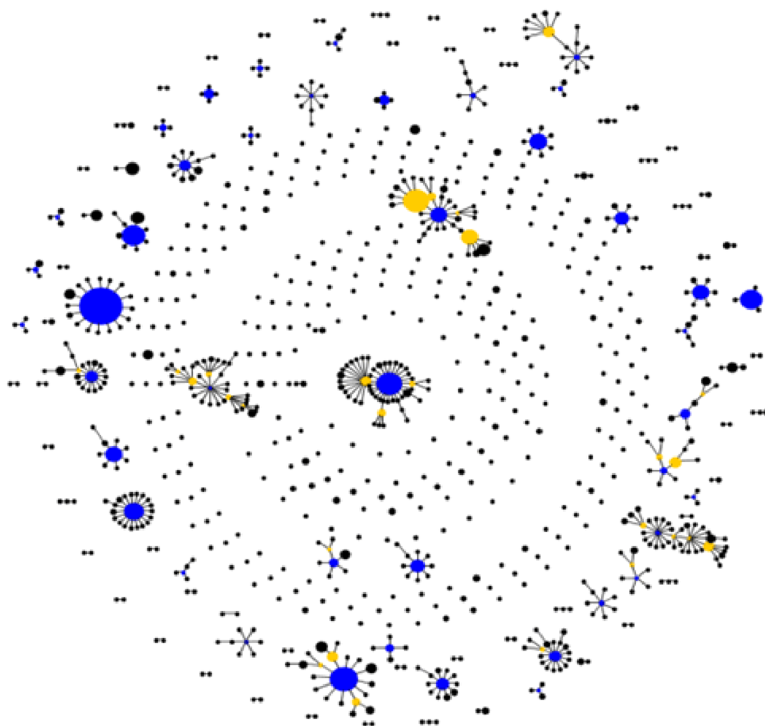


Figure 18. Example eBURST diagram depicting the relationship between closely related bacterial isolates based on MLST. Sequence types (STs) are represented by dots, the larger the dot - the more it is represented in the population. Black dots; STs, blue dots; primary founders, yellow dots; subgroup founders.

4.133 Genotyping and population mapping of keratitis isolates

Stewart et al[231] studied 63 *P. aeruginosa* isolates collected between 2003 and 2004 from patients with keratitis to investigate whether specific *P. aeruginosa* clones are associated with the disease. Isolates taken from patients with *P. aeruginosa* associated keratitis were genotyped using the AT methodology as previously described. eBURST population analysis, comparing keratitis isolates to a wider collection of *P. aeruginosa* from various non-ocular sources, identified various markers in a subpopulation of *P. aeruginosa* associated with keratitis that were in strong disequilibrium with the wider *P. aeruginosa* population. There was a significantly higher proportion of isolates with the *oriC1* allele within the keratitis group compared to the isolates taken from non-keratitis sources. These *oriC1* isolates were much more likely to be *exoU*+ve and *exoS*-ve in the keratitis group compared to the non-keratitis group. The keratitis isolates were also shown to be more frequently associated with *exoU,* glycosylated but unmodified flagellin and the absence of the nonhaem catalase KatN (chapter 4.121). One isolate (39016) was selected for further genome sequencing. This was because it represented the commonest clone (clone D) and serotype (O11). Strain 39016 was shown to have a novel type 4 pili gene cluster *pilA* gene. Type 4 pili are associated with adhesion and twitching motility (chapter 4.1221), which is thought to be important in keratitis.[194, 196] The majority of clone D isolates and some other O11 isolates carried this novel *pilA* gene. Winstanley et al[205] reported that 90% of 63 keratitis isolates exhibited better twitching motility than strain PA14.

Stewart et al[231] concluded that the similar genetic characteristics of the keratitis isolates suggests that a subpopulation of *P. aeruginosa* may be adapted to

causing corneal infections. It is unclear however, if these features are consistent temporally or represent a feature of the particular time period chosen for sampling.

4.2: Chapter aim

This chapter aims to investigate whether the genetic features of the isolates investigated by Stewart et al[231] are consistent temporally with *P. aeruginosa* isolates associated with keratitis. A population genotype of *P. aeruginosa* keratitis isolates from a later time period; 2009-2010 will be compared to the isolates described by Stewart et al.[231]

4.3 Methods

4.31 Genotyping and population mapping of P. aeruginosa

P. aeruginosa isolates were taken from patients presenting with bacterial keratitis between 2009 and 2010 (chapter 2.11). DNA was extracted from the isolates using the Promega wizard kit (chapter 2.31). Genotyping was conducted using the AT genotyping system (Alere Technologies, Jena, Germany).[229, 231] Components of the AT system are described in appendix B2. Analysis of 13 SNPs based on the conserved genome, and three variable markers (flagellin types a or b and the mutually exclusive type III secretion exotoxin genes *exoU* or *exoS*) was performed as follows:

1) Linear amplification and biotin labelling

A PCR master mix was prepared with: 4.9 µL of B1 (2x Labelling Buffer), 0.1 µL of B2 (DNA Polymerase) and 0.5-2 µg of the extracted DNA suspended in 5 µL

of water. PCR conditions: 300 sec at 96 °C, 50 cycles of 20 sec at 62 °C, 40 sec at 72 °C and 60 sec at 96 °C. 90 µL of buffer C1 was added.

2) Array Tube hybridisation

General precautions with Array Tubes: Plastic Pasteur pipettes were used with flexible tips and care was taken not to touch the Array Tube surface.

Pre-washing of the Array Tubes: 500 µL of ultrapure water was added to each tube. Tubes were incubated in the thermomixer at 60 °C, 550 rpm for 2 minutes. Water was then removed from the tubes. 200 µL buffer C1 added to each tube. Tubes were incubated in the thermomixer at 60 °C, 550 rpm for 2 minutes. Buffer C1 was removed and discarded.

Hybridisation: Amplified DNA from step 1 was transferred to a prepared Array Tube which was then incubated for one hour at 60 °C and 550 rpm.

Washing steps: The hybridisation mixture was removed and 500 µL of buffer C2 was added and discarded from the tubes. A further 500 µL of buffer C2 was added and the tubes were incubated for 5 minutes at 30 °C, 550 rpm followed by removal of the washing solution. This step was repeated once.

Addition of HRP conjugate: 100 µL C3/4 (containing Streptavidin-Horseradish Peroxidase (HRP) was added to each tube followed by incubation for 10 minutes at 30 °C and 550 rpm. C3/C4 was then removed and 500 µL of buffer C5 was added and removed. This step was repeated.

Staining of bound HRP-conjugate: 100 µL of reagent D1 (containing a substrate for Horseradish Peroxidase) was added to each tube which was then incubated at room temperature without agitation for 10 minutes.

3) Data Acquisition

The final washing solution was removed from the ATs and 100 µL of 3,3'5,5',-tetramethylbenzidine staining solution was added. Data was acquired from the chip by inserting the AT containing the hybridised and stained products into an AT Reader. Images were acquired using the IconoClust-AT software (CLONDIAG Chip Technologies GmbH). Acquired images were composed of a series of dots (see figure 16b for example), which was converted into a 4 digit code, as described in chapter 4.131.

Isolates with 4 digit codes found more than once in the study were designated a clone 'type' depicted by a capital letter (e.g. A) or a letter followed by a number (e.g. A3). Isolates with 4 digit codes that were found only once in the study were recorded as 'single' and those not previously identified as 'novel'. The genotypic relationship between our isolates and *P. aeruginosa* isolates from other sources was assessed using the 4 digit code using the eBURST (v3) algorithm (Imperial College London, UK).[232,233] 4 digit codes were entered to the eBURST website (http://eburst.mlst.net/v3/enter_data/comparative/mlst/default.asp) and a comparison was made to 322 *P. aeruginosa* isolates taken from environmental and other non-ocular sources. [231,229, 234,235,236]

4.32 Distribution of regions of difference

Background: In the previous study of the 2003-4 isolates, Stewart et al[231] selected isolate 39016 for further genome sequencing. This was because it represented the most abundant clone in their study (clone D) and the most common serotype (O11), it occupied a central location within the major cluster of isolates, and it was associated with a severe keratitis. In addition, AT data suggest

that it lacks many of the accessory genome genes represented on the microarray. Hence, they hypothesized that its accessory genome carries novel genomic islands shared with other keratitis isolates. From this they identified accessory genome regions which differed from the laboratory PAO1 strain and designed assays to screen for regions of differences (ROD).

Methods: Indicative PCR assays designed by Stewart et al[231] were used to determine the distribution of the RODs showing a frequency > 15% amongst the 63 isolates of the 2003-2004 study (ROD 1, 15, 16, 17, 18 and the novel *pilA* gene) amongst the 60 isolates from the current study. PCRs for each of these RODs were multiplexed with an assay for the *oprL* gene as an internal control. Table 13 lists individual primer details. *P. aeruginosa* isolate strain number 039016 was used as a positive control. All reactions were conducted with initial denaturation at 94 °C (5 minutes), followed by 25 cycles of denaturation (92 °C, 3 mins), annealing (58 °C, 1 minute) and elongation (72 °C, 2 mins), with final elongation at 72 °C (10 mins).

Statistical analyses: Independent data comparing genetic features of keratitis isolates in a temporal manner or comparing features of keratitis isolates with non-keratitis isolates were assessed by chi square double classification with one degree of freedom.

4.4 Results

Distribution of clone types amongst 2003–2004 and 2009–2010 collections

60 keratitis-associated *P. aeruginosa* isolates were collected by the MOG (2009 to 2010). DNA was extracted and successful AT image acquisition was performed in all of them. Appendix D describes the clone type attributed to each isolate in the

study. 36 (60%) of the isolates analysed in this study were assigned to an existing clone type in the database of 322 strains. This compares with 33 of 63 (52%) isolates from the 2003 to 2004 collection.[231] Clone types that did not yield a match in the published database were assigned as 'novel' clone types. Nearly 23 novel clone types (representing 25 of 60 isolates) were identified in this study compared to 19 novel clone types (representing 30 of 63 isolates) in the previous study from 2003 to 2004. The combined prevalence for the six most common clone types (clones A, B, C, D, I and V) was similar in the two collections [27 of 60 (45%) in 2009–2010 compared to 24 of 63 (38%) in 2003–2004]. Among keratitis isolates, one novel clone type (C429) was identified at both time points.

Keratitis isolates within the wider *P. aeruginosa* population structure

When keratitis isolates were examined within the wider *P. aeruginosa* population structure, it was possible to identify two major clusters of *P. aeruginosa*; cluster 1 and cluster 2 (figure 19). 86 of 123 (71%) keratitis-associated isolates were present within cluster 1, representing 39% (86 of 222) of all isolates in this cluster. Forty-seven of 63 (75%) isolates from 2003 to 2004 and 39 of 60 (65%) of the 2009–2010 isolates were found in this cluster. In comparison, 135 of 322 (42%) of the non-keratitis isolates were within cluster 1, which is significantly reduced ($p = 0.001$) compared to the percentage of keratitis isolates within the cluster.

Table 13. List of oligonucleotide primers used to detect regions of differences (RODs)

Primer	Sequence	Size (bp)	Target gene /ROD[a]	Ref
PAL1	ATGGAAATGCTGAAATTCGGC	504	*oprL*	[237]
PAL2	CTTCTTCAGCTCGA		*oprL*	
ORF5228F	GTCATGCCCACAAACTGATG	325	ROD16	[231]
ORF5228R	ACCTTGGTGGACCGCTTAC		ROD16	
ORF6116F	TCGAATGTGAAGTGCCTCAG	218	ROD18	[231]
ORF6116R	GTAACGGATTTCGGTGTTGC		ROD18	
ORF4339F	AACTCGCAATCCACCGTATC	150	ROD15	[231]
ORF4339R	GATCCGTCCTCCTGTTTCAA		ROD15	
ORF5388F	TGTTCATGGACATGGAGGAA	326	ROD17	[231]
ORF5388R	CAGCTCGTTCTGGTCTTCG		ROD17	
ORF265F	GTGGGTTTGCAAAAGCGTAT	234	ROD1	[231]
ORF265R	CACCTCTTCAGGTGTGCTGA		ROD1	
novPilAF	CGGGTTCCAGTTTGTTGACT	184	*pilA*	[231]
novPilAR	CAGCCACCATTAACATCACG		*pilA*	

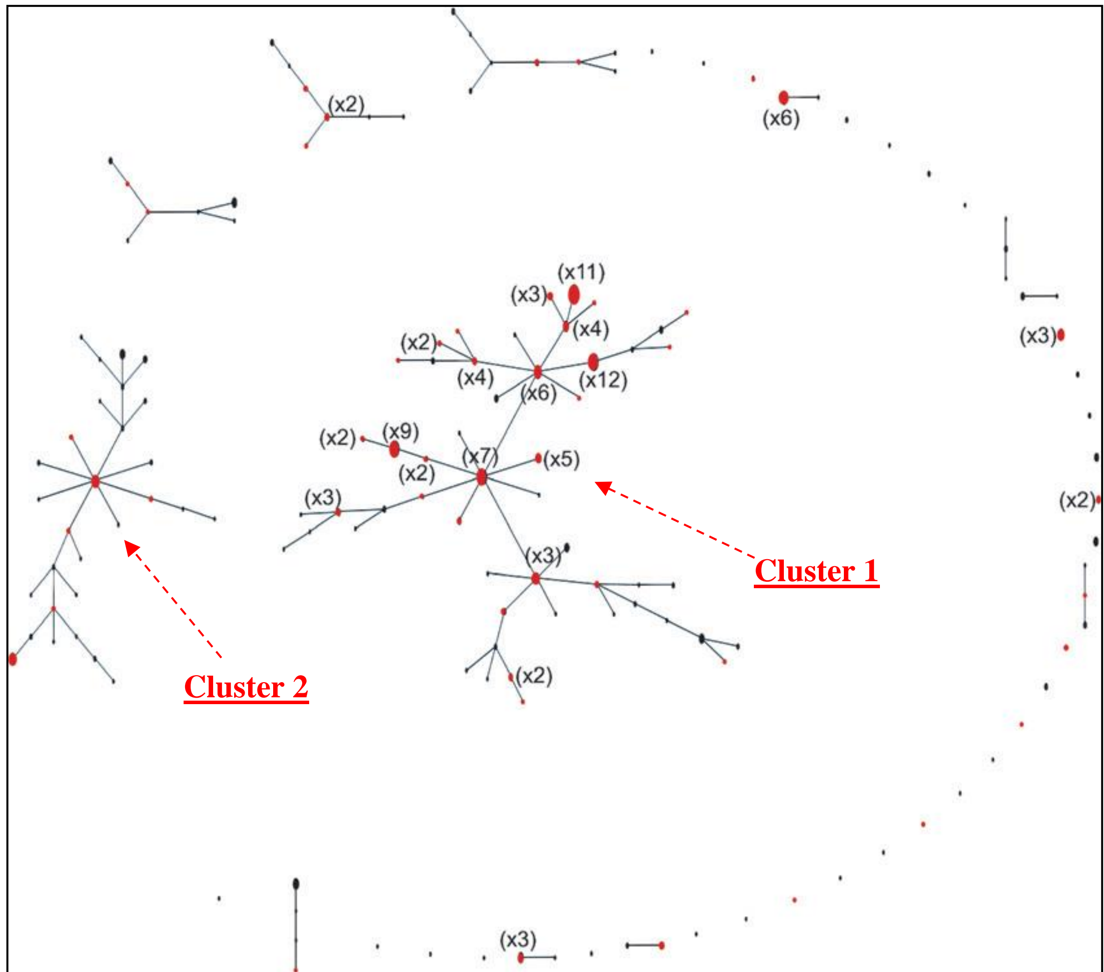


Figure 19. Keratitis isolates within a wider *P. aeruginosa* population structure. The diagram shows an eBURST representation of 123 keratitis-associated *P. aeruginosa* isolates (red spots) among a total of 445 strains. The number of isolates for each clone type that was recovered is indicated in brackets. Where no number is given, only one isolate of that type was identified. Two clonal complexes are seen indicated as cluster 1 and cluster 2. 71% of keratitis isolates fall within cluster 1.

Comparison of prevalence of genomic islands and variable genes between the 2003-2004 and 2009-2010 isolate collections

All 60 of the 2009–2010 keratitis isolates carried the PAGI-1 genomic island, a common genomic island found in 85% of clinical isolates.[238] On the AT chip, PAGI-2- and PAGI-3-like genomic islands were represented by 10 hybridisation signals.[229, 234] Analysing both 2003-4 and 2009-10 groups together showed a similar prevalence of the genomic island compared to non-keratitis isolates; 65 of 123 (53%) keratitis isolates lacked PAGI-2/3-like genomic islands compared with 159 of 322 (49%) non-keratitis *P. aeruginosa*. PAPI-1, PAPI-2 and pKLC102 represent a family of genomic islands that carry virulence genes absent in the commonly used laboratory strain *P. aeruginosa* PAO1. AT markers pKL-1 and pKL-3 represent conserved domains of this family of genomic islands.[229, 234] Sixty-seven of 123 (55%) keratitis isolates did not show hybridisation for either marker pKL-1 or pKL3 compared to 122 of 322 (38%) non-keratitis isolates (P = 0.05). *P. aeruginosa*-type flagellins vary because of the presence of a glycosylation island[239] that can be present as either a longer insert encompassing 14 open reading frames (ORFs), or as a shorter version with a 5.4-kb deletion.[240] Twenty of 123 (16%) keratitis isolates carried the full length glycosylation island (12 of 63 isolates in 2003–2004 and 8 of 60 isolates in 2009–2010) and 61 of 123 (50%) carried the truncated version. This compares with 28% and 35% of non-keratitis isolates carrying the full length and truncated glycosylation island, respectively.[231] Carriage of the variable gene PA2185 encoding the nonhaem catalase KatN was higher (25 of 60; 42%) in the second isolate collection compared with the first isolate collection (18 of 63; 29%), but this increase was not significant (v2 = 2.318). Carriage of PA2185 is significantly lower (P = 0.001)

among keratitis isolates (43 of 123; 35%) than amongst the non-keratitis collection (188 of 322; 58%). Carriage of the *exoU* island A[224] is associated with the non-PAO-1 type *oriC1* allele in keratitis isolates.[231] *exoU*-positive strains continued to show significant (P = 0.001) association with the presence of *oriC1* in the 2009–2010 isolate cohort, whereas *exoS*-positive strains do not show association with either *oriC* allele. When we included all 120 keratitis isolates (three isolates were negative for *exoS* and *exoU*) from both studies, the association between *exoU* and *oriC1* allele continued to be significant (P = 0.001).

Distribution of ROD associated with *P. aeruginosa* keratitis strains 2003-04

In the previous study of the 2003–2004 isolates,[231] isolate 039016 was selected for genome sequencing and PCR assays were developed to analyse the distribution of 10 ROD among the 63 keratitis isolates. Table 14 shows that among the 60 keratitis isolates from 2009 to 2010, the prevalence of four of the ROD and the novel *pilA* showed significant reduction compared to the 2003–2004 collection (P = 0.05). The only exception was ROD16 (26.7%). To establish whether ROD16 might be a specific feature of keratitis-associated *P. aeruginosa*, contemporary blood culture isolates of *P. aeruginosa* were analysed. The prevalence for ROD16 amongst the blood culture isolates was 22.2%, suggesting that carriage of this region was not something particular to isolates associated with keratitis.

Table 14. Prevalence of ROD of interest among keratitis strains isolated between 2003–2004 (Stewart et al., 2011) and 2009–2010 (this study)

		2003-04 study (n = 63)		2009-10 study (n = 60)	
Region	**size(kb)**	+ve	%	+ve	%
ROD16	39.7	19	30.2	16	26.7
ROD18	> 13.5	19	30.2	8	13.3*
ROD15	> 45	14	22.2	1	1.7*
ROD17	37.2	12	19.1	3	5.0*
ROD1	34.7	11	17.5	1	1.7*
Novel		17	27.0	2	3.3*

+ve = number of isolates positive for ROD. % = prevalence of ROD in group of isolates. *Statistically significant (P= 0.05; chi-squared test) deviations from previously determined prevalence of ROD of interest.

4.5 Discussion

Various approaches have been used to define the population structure of *P. aeruginosa* and to identify an association between strain types and environmental origin or particular types of infection. Using a combined analysis of amplified fragment length polymorphism (AFLP), serotype, pyoverdine type and antibiograms, Pirnay et al[241] concluded that population diversity in river water reflected the wider population diversity of *P. aeruginosa* and that environmental and clinical isolates are indistinguishable. A combination of phenotypic and genotypic characteristics used in a larger survey reached similar conclusions.[242] In contrast, a study using MLST indicated that oceanic isolates were divergent from the general *P. aeruginosa* population.[243] AT genotyping has been applied to collections of isolates of clinical relevance, particularly in chronic infections associated with cystic fibrosis[244] and chronic obstructive pulmonary disorder.[230]

Although dominant clones are a feature in these populations, evidence for an association between a subgroup of *P. aeruginosa* clones and a specific type of infection has only been reported in the previous study AT genotyping of keratitis isolates by Stewart et al.[231]

To determine whether this association of a clonal subgroup with disease was a unique occurrence among UK keratitis isolates collected between 2003 and 2004, rather than an inherent feature of isolates associated with this disease, the study was replicated on a further set of 60 isolates obtained 5 years later from the same contributing hospitals. Our results show that there was a similar cluster to that observed previously, revealing that a subgroup of keratitis-associated *P. aeruginosa* strains was a feature of both collections when analysed separately or when combined (n = 123). There were some minor variations between the two time points. Differences were observed in the dominant clone types (type A in 2009–2010 vs. type D in 2003–2004). There was also a reduction in the proportion of keratitis isolates falling within the core keratitis cluster (cluster 1) between the time points (40% in 2009–2010 vs. 48% in 2003–2004). However, overall 71% of keratitis isolates belonged to a core keratitis cluster (cluster 1; figure 19). Although the carriage of the *exoU/S* was not included in the eBURST analysis, all of the *exoU* positive keratitis isolates (66 of 123) belonged to cluster 1. This cluster also includes 19 isolates carrying the *exoS* gene. However, 35 of the 36 keratitis isolates not within cluster 1 carry the *exoS* gene.

In the previous study by Stewart et al,[231] RODs were identified between keratitis isolate 039016 (AT clone type D; serotype O11; poor clinical outcome) and strain PAO1. The carriage of these regions as indicated by PCR assay was lower in the more recent collection of isolates. It is likely that this is because of a

lower number of Clone D isolates in the more recent collection and that these RODs were largely associated with Clone D specifically, rather than a general features of the cluster. The exception was ROD16. However, the similar prevalence of this ROD amongst blood culture isolates of *P. aeruginosa* suggests that ROD16 is not a particular feature of keratitis associated isolates. Previously identified characteristics associated with the core keratitis cluster described by Stewart et al[231] were confirmed in the current study. The keratitis-specific subpopulation strains carry the *oriC1* allele, *exoU*, and a truncated version of the flagellin glycosylation island, but are less likely to carry the gene encoding the nonhaem catalase KatN (. As previously noted, carriage of the *exoU* gene was significantly associated with the *oriC1* allele.

The AT genotyping scheme has also been used to analyse strains from diverse backgrounds, indicating the presence of dominant clones that are widely distributed.[229, 234] A recent study using AT typing reported the presence of several extended clonal complexes (ecc) that were nonuniformly distributed in freshwater sources of varying water quality, suggesting that the population dynamics of *P. aeruginosa* may be shaped by environmental rather than clinical factors.[245] Isolates of the divergent eccB were the most frequently sampled from various environmental water sources, prompting the suggestion that this clonal complex represents a ‘water ecotype’ better adapted to environmental water than other *P. aeruginosa*. Interestingly, an *exoU+/exoS-* genotype is a feature within this eccB group. In this study, the core keratitis cluster was found to include clone types (such as A, B, D and I) that are eccB clone types.[245] The eccB group also includes serotypes O11, O10 and O8 which feature prominently amongst the core keratitis cluster. For 78 isolates, we had clinical data regarding the use of contact lenses.

Although the differences were not statistically significant, a greater proportion of core keratitis cluster isolates were associated with contact lens use (72%, 56 of 78) than for isolates not within the core cluster (28%, 22 of 78). A larger sample size would be needed to test whether this association is significant.

Full gene sequencing is being increasingly performed in *P. aeruginosa* genetic studies. [246, 247] Gene sequencing of the keratitis isolates used in this study could provide a more complete understanding of the *P. aeruginosa* genome and its involvement in keratitis.

4.6 Conclusion

It appears from this study, and the study by Stewart et al,[231] that there is a sub-set of *P. aeruginosa* isolates that are associated with bacterial keratitis in the UK. These isolates have been found to be related to the eccB clonal complex associated with adaptation to survival in environmental water,[245] which is consistent with the notion that aquatic environments are integral to the transmission dynamics of *P. aeruginosa*, in the context of bacterial keratitis. However, the link between specific genotypes and clinical outcome or risk factors is not clear. Further analysis of clinical data and studies involving additional sets of patients for verification of this hypothesis will provide a clearer picture, helping to link genetic features with evidence-led clinical management of *P. aeruginosa* keratitis.

CHAPTER 5: ANTIMICROBIALS IN KERATITIS

5.1 Introduction

5.11 How do antimicrobials work?

Antimicrobials are a class of drug that kills or prevents the growth of microorganisms. The efficacy of an antimicrobial is dependent on its pharmacodynamics and pharmacokinetics. Pharmacodynamics is the study of the effects of drugs on the body, or on microorganisms within the body. It also includes the mechanisms of drug action and the relationship between drug concentration and effect. Pharmacokinetics is concerned with the fate of substances administered externally to a living organism. It can be subdivided into the following four processes; absorption, distribution, metabolism, and excretion,

5.112 Pharmacodynamics

Antimicrobial pharmacodynamics is measured by determining its MIC, which is defined as the lowest antimicrobial concentration that will inhibit overnight growth of bacteria (chapter 2). The MIC_{90} is a descriptive statistic estimating the antimicrobial concentration which will inhibit the growth of 90% of isolates and the MIC_{50} is the concentration which inhibits 50% of isolates.

There is good evidence demonstrating the relationship between the MIC of topically applied antimicrobials and clinical outcome in bacterial keratitis for certain bacteria. Kaye et al[181] described the relationship between clinical outcome of patients with bacterial keratitis (defined by the ratio of healing time to ulcer size) and the MIC of a particular antimicrobial agent. General linear multivariate

modelling showed a significant association between the MIC of the antimicrobial prescribed and clinical outcome for *Pseudomonas* spp., *S. aureus* and *Enterobacteriaceae.* A significant association was not seen for CNS, suggesting that CNS may not necessarily be pathogenic in bacterial keratitis.

The MIC is used to determine the susceptibility or resistance of an antimicrobial, by comparing it to a set of standard MICs based on the safe achievable concentrations of antimicrobial in the serum (chapter 5.15). It is important to note that systemic interpretive MIC breakpoints are likely to be inappropriate for the topical ophthalmic use of antimicrobials. The clinical outcome of a corneal infection to a given topically applied antimicrobial, is not only dependent on the MIC of the infecting bacteria, but is also critically dependent on the achieved corneal antimicrobial concentration and bioavailability (i.e. its pharmacokinetics, see next section). This is recognised by BSAC,[118] who state the following in their antimicrobial testing guidelines; "*MIC breakpoints specific for topical antibiotics are not given because there are no pharmacological, pharmacodynamic or clinical response data on which to base recommendations. Relevant data would be gratefully received.*"

5.113 Pharmacokinetics

When evaluating pharmacokinetics one must consider the body spaces between which drugs pass and within which drugs are distributed. Ocular pharmacokinetics is concerned with (a) the tear film and conjunctival fornices, (b) the anterior chamber, (c) the vitreous cavity and (d) the retro-orbital space. Most topical ophthalmic drugs exhibit first order kinetics; where the absorption rate and elimination rate of the drugs vary directly with the drug concentration.[248] The

drug half-life in first order kinetics is therefore constant regardless of the amount of drug that is present.

Upon administration of topically applied drops to the ocular surface, precorneal factors and anatomical barriers negatively affect the bioavailability of topical formulations.[249] Precorneal factors include solution drainage, blinking, tear film and lacrimation. The tear volume is estimated to be 7 μl, and the cul-de-sac can transiently contain around 30 μl of the administered eye drop. The tear film, however, displays a rapid restoration time of 2–3 minutes, and most of the topically administered solutions are washed away within just 15–30 seconds after instillation.

Topically applied drug may penetrate into the eye via the cornea, the conjunctiva or the sclera. In practice, however, the vast majority of topical drugs penetrate via the cornea.[249] The cornea is composed of several layers with different physiological and anatomical functions (chapter 1.2). The corneal epithelium is the most important layer when considering the penetration of topically applied drugs into the eye.[250] During the maturation of the corneal epithelium, the cells become flatter and form tight junctions in the intercellular space. These tight junctions, known as zonula occludens, are located only in the most apical surface cell layers, providing the diffusional barrier for drug absorption from the tear film to the anterior chamber. The corneal epithelium poses a significant resistance for permeation of topically administered hydrophilic drugs.[251] The presence of zonula occludens restricts paracellular drug permeation from the tear film into the cornea. The highly hydrated structure of the corneal stroma poses a significant barrier to permeation of lipophilic drugs. The endothelium possesses leaky junctions that facilitate the passage of

macromolecules between the aqueous humour and stroma. This layer is therefore not as important as the epithelium and stroma as a barrier for drug penetration.[249] In bacterial keratitis there is usually an ulcer present so that neither the epithelium or its basement membrane are present. This would allow greater penetration of antimicrobials directly into the stroma.

Trans-corneal penetration of topically applied drug can be through passive diffusion or active transport mechanisms. Passive diffusion is driven by the physico-chemical parameters that determine the partitioning and diffusion of the drug in the cell membrane. Lipophilicity is thought to be the critical feature of a drug that determines its passive diffusion through the cornea.[250, 252] Compounds that are more lipophilic, as a general rule, penetrate the cornea more readily. The optimal lipophilicity for corneal permeation has been estimated between 2–3 logD values.[253] Molecular weight, pH and stability in solution all may play a part in the passive permeation of a drug. Active transport of drug through the cornea requires the expression of transporters in the epithelium.[250] Corneal transporters that have the highest impact on drug permeation are located in the most apical surface of the corneal epithelium.[254] Transporter expression in other locations in the cornea such as wing cells and basal cells of the epithelium, keratocytes and corneal endothelium are important in physiology, but are thought to be less relevant in pharmacokinetics. Transporters that have been described in the human corneal epithelium include the SLC6 neurotransmitter transporter family[255] and the SLC7 cationic amino acid transporter y+ system.[256] Since corneal permeability is the sum of passive diffusion and active transport, the relative impact of active transport depends on the background level of passive diffusion. Active transport mechanisms may have a higher impact on the permeation of hydrophilic

compounds than that of the lipophilic drugs, due to the low passive diffusion of hydrophilic drugs.

5.12 Drugs used to treat keratitis

5.121 History of antimicrobial use in keratitis

In the 1940s, the vast majority of Gram-positive and Gram-negative bacteria were found to be susceptible to the early antimicrobials penicillin, streptomycin, and tetracycline.[257] For this reason these antimicrobials were popular choices for topical treatment of bacterial keratitis. The overuse of penicillin, however, led to the selection of resistant strains. By the end of the 1950s, *S. aureus* had acquired resistance to virtually all available systemic antimicrobials, including erythromycin and the tetracyclines.[258] In 1960 meticillin was introduced, a β-lactam antimicrobial that is not inactivated by the enzyme β-lactamase.[259] A host of other β-lactam antimicrobials with similar properties, including oxacillin, nafcillin, and cefazolin, soon followed.[260]

At the end of the 1960s, gentamicin was developed from the old aminoglycoside streptomycin, because of the upsurge in *P. aeruginosa* infections (susceptible to gentamicin but resistant to streptomycin). Over the next decade, other aminoglycosides, tobramycin and amikacin, were introduced and commercial drops of tobramycin and gentamicin became available, which become popular for the treatment of bacterial keratitis.[257] In the 1970s and 1980s there was extensive development of new β-lactams to overcome the problems of resistant Gram-negative bacterial infections and of aminoglycoside toxicity. The cephamycins (cefoxitin and cefotetan), the "third-generation" cephalosporins (ceftriaxone, ceftazidime, cefotaxime and others), and the carbapenems

(imipenem) were all used for systemic treatment.[261] The development of an old class of antimicrobials, the quinolones, in the 1990s, provided for the first time a class of drugs with broad Gram-positive and Gram-negative activity and little corneal toxicity.[257] The second generation fluoroquinolones, ciprofloxacin and ofloxacin have been found to be as effective for bacterial keratitis as fortified drops of older agents and are freer of the problems of corneal toxicity and short shelf-life. Third generation (levofloxacin) and fourth generation fluoroquinolones (moxifloxacin and gatifloxacin) are now FDA approved for the treatment of bacterial keratitis.[262]

5.122 Fluoroquinolones

Fluoroquinolones work by inhibiting DNA gyrase (also known as topoisomerase II) and topoisomerase IV, enzymes necessary in bacterial DNA synthesis. Second generation fluoroquinolones, ciprofloxacin and ofloxacin, are widely used in treating bacterial keratitis. They offer a great potency against Gram-negative bacilli (including *P. aeruginosa)*, moderate activity against *S. aureus* and little activity against Streptococci and the pneumococci. Despite the success of the first- and second-generation fluoroquinolones, there has been a trend (based on systemic breakpoints) towards an in increase in resistance of both *S. aureus*[263] and *P. aeruginosa*.[264] Further molecular modifications of the fluoroquinolones in 2000s lead to the development of the third-generation levofloxacin, and the fourth generation moxifloxacin and gatifloxacin. These agents have greater potency against Gram-positive bacteria, in particular the Streptococci. The later generation fluoroquinolones unfortunately have not been a treatment panacea because of the emergence of resistance (albeit based on systemic breakpoint data).[265, 266] Park et al.[267] showed a rate of 2% resistance (based again on systemic breakpoint data) to

moxifloxacin and 5% to gatifloxacin in isolates of normal bacterial ocular flora. Sueke et al[104] showed a rate of 2% resistance using systemic breakpoint data to moxifloxacin and 16% to ciprofloxacin in *S. aureus* isolates from patients with bacterial keratitis. A number of pharmacokinetic studies have shown moxifloxacin to have superior corneal penetration compared to the other fluoroquinolones.[268-270] For example, in a rabbit endophthalmitis model[270] aqueous levels of levofloxacin were 9 mg/L, and moxifloxacin was 43 mg/L after topical administration. The greater lipophilicity of moxifloxacin compared to the other fluoroquinolones may explain this phenomenon. Besifloxacin is a novel fluoroquinolone for topical ophthalmic use recently approved by the US Food and Drug Administration (FDA) for the treatment of bacterial conjunctivitis.[271] Besifloxacin appears to have a broad spectrum of activity against aerobic and anaerobic bacteria, possibly due to its cyclopropyl group and chloride substituent at C-8 improving its activity against DNA gyrase and topoisomerase IV enzymes. Besifloxacin has been shown to be active against both Gram-positive (*S. aureus, S. pneumoniae, Corynebacterium* and *Propionibacterium acnes*) and Gram-negative organisms (*H. influenzae*, *Moraxella*, *E. coli*, *Neisseria gonorrhoeae* and *P. aeruginosa*). Recent studies have found besifloxacin to have good pharmacokinetic parameters *in vitro*[272] as well as excellent efficacy in animal models of keratitis, compared to fourth generation fluoroquinolones.[273, 274]

5.123 Aminoglycosides

Aminoglycosides such as gentamicin and tobramicin, are often used to treat bacterial keratitis. They have a broad range of bactericidal activity against many bacterial species, particularly Gram-negative rods. They have an affinity to bacterial 30S and 50S ribosomal subunits producing a non-functional 70S

initiation complex resulting in an inhibition of protein synthesis. They are sometimes given in combination with predominantly Gram-positive antimicrobials. Their use is limited by their associated corneal toxicity.[275] Sueke et al[104] showed 4% of isolates to be resistant to gentamicin against *S. aureus* and *P. aeruginosa*, using systemic breakpoints. 4% of *P. aeruginosa* isolates and 0% of *S. aureus* isolates were resistant to amikacin. Gentamicin has, however, been shown to have poor corneal penetration which may be due to the hydrophilic nature of the compound. Baum et al[276] showed that the concentration of gentamicin in the aqueous at 1 hour is only 1 mg/L, which is lower than the suggested MIC using systemic breakpoints.

5.124 Cephalosporins

Cephalosporins have a broad spectrum of activity, including effective action against *Haemophilus* species. They contain a β-lactam ring similar to penicillins but have the advantage of being resistant to the penicillinases. They inhibit bacterial cell wall synthesis and are well tolerated topically. The 1st generation cephalosporins include cephazolin, 2nd generation cefuroxime and 3rd generation ceftazidime. Cefuroxime is often used in combination with an aminoglycoside for the empirical treatment of suspected bacterial keratitis. Sueke et al[104] showed cefuroxime and ceftazidime had high MICs against *S. aureus* and *P. aeruginosa* suggesting a significant degree of antimicrobial resistance, however, systemic breakpoints were not available to formally assess this. The poor corneal penetration of cefuroxime maybe due to due to it being hydrophilic in nature.[277]

5.125 Meropenem

Meropenem is a broad-spectrum carbapenem that is currently FDA approved to treat skin infections, intraabdominal infections and bacterial meningitis.[278]

Meropenem is a β-lactam antimicrobial, working through bacterial cell wall inhibition. The antimicrobial, however, differs from other β-lactam antimicrobials, as it is chemically stable to hydrolysis by the most common β-lactamases (chapter 5.131).[279] It has activity against Gram-positive and Gram-negative pathogens, including extended-spectrum lactamases (ESBL) and AmpC-producing *Enterobacteriaceae*. Sueke et al[104] showed meropenem to have wide coverage against both Gram-positive and Gram-negative microorganisms, where only one of the 772 isolates tested (*P. aeruginosa*) was resistant using systemic breakpoints. Corneal pharmacokinetics of meropenem are not yet known, however intravitreal meropenem in a rabbit model of endophthalmitis[280] did not show any evidence of toxicity. Similarly intravenous meropenem prior to cataract surgery showed penetration of the drug into the anterior chamber with no notable side effects.[281]

5.126 Combination therapy in bacterial keratitis

As opposed to single therapy, an antimicrobial combination offers a broader spectrum of activity and may reduce selective pressures. This may be of particular importance for the fluoroquinolones, as increasing resistance has been reported in *S. aureus* and *P. aeruginosa* isolates from cases of bacterial keratitis.[111, 263-266] An often overlooked reason for combination therapy, however, is not for providing a broader spectrum but for an increased antimicrobial effect. In particular, combination therapy may result in synergy as occurs, for example, with the combination of penicillin and gentamicin when used in the treatment of enterococcal endocarditis.[282, 283] This synergistic effect can be explained by the increased ease of gentamicin passage into the bacterial cell, due to cell wall disruption caused by the action of penicillin. Conversely, combinations of

antimicrobials may be antagonistic, as occurs with the combination of chloramphenicol and penicillin in the treatment of pneumococcal meningitis.[284] The presumed reason for this antagonism is; bacterial growth is reduced by chloramphenicol, this prevents penicillin, which requires a dividing and growing organism, from having its full effect on cell wall synthesis inhibition. It is important therefore not to use combination therapy which may have inhibitory or antagonistic effects.

Antimicrobial combinations can be assessed *in vitro* by checkerboard, time-kill and E-Test methods. All methods involve determining the ratio of the MIC of each antimicrobial when tested alone, compared to when the antimicrobials are combined (chapter 5.42), which determines if the combination is additive, synergistic, indifferent or antagonistic. Suzuki et al[285] investigated various antimicrobial combinations against isolates taken from patients with keratitis. They showed that the combinations of levofloxacin/cefmenoxime, and gatifloxacin/cefmenoxime were additive in over 70% of isolates tested. No consistent synergistic or antagonistic effect was observed with the combinations used.

5.13 Antimicrobial resistance

Antimicrobial resistance is the development of resistance in a microorganism, to an antimicrobial to which it was previously sensitive. A wide range of biochemical and physiological mechanisms may be responsible for resistance. Antimicrobial resistance is a consequence of the overuse of antimicrobials and develops when a microorganism mutates or acquires resistance genes. Increasing resistance could compromise the utility of a valuable class of antimicrobial agents,

which emphasises the importance of the careful use of antimicrobials, in appropriate patients, at the correct dose.

To determine whether an isolate is resistant or susceptible to an antimicrobial, the MIC (chapter 5.112) is compared to a set of standard MICs based on the safe achievable concentrations of antimicrobial in the serum. These standards are set by the Clinical and Laboratory Standards Institute (CLSI) in the USA and BSAC in the UK (table 15). As mentioned in chapter 5.112, interpreting resistance and susceptibility needs to be done with caution, as currently there are no standards for topical ocular therapy that relate to the concentrations of antimicrobial in ocular tissue. For example, Sueke et al[104] found the range of MICs for ciprofloxacin against 140 *P. aeruginosa* isolates to be 0.016 to 6.0 mg/L. Using the breakpoint figure of 1.0 mg/L from BSAC, which was calculated from systemic data, 98% of isolates were susceptible to ciprofloxacin.

The antimicrobial resistance mechanisms of *S. aureus* and *P. aeruginosa* will now be discussed.

5.131 S. aureus resistance

S. aureus is well known for its resistance to the β-lactam group of antimicrobials including penicillins, cephalosporins, carbapenems, and monobactams.[257] *S. aureus* resistance to penicillin is attributed to the production of penicillinase (a class A β-lactamase), an extracellular enzyme that hydrolyses the amide bond of the β-lactam ring of penicillin, yielding an inactive compound. Novick et al[286] demonstrated in 1971, that the genes responsible for the synthesis of penicillinase and its regulation were extrachromosomal and carried by a plasmid.

Meticillin was the first antimicrobial specifically tailored to counteract a bacterial resistance mechanism, initially being invulnerable to the attack of

penicillinase. Occasional strains of MRSA were first detected in 1961 very soon after its introduction but these were only resistant to β-lactam antimicrobials. MRSA strains in Australia in the late 1970s, however, were found to be resistant to other antimicrobial classes. The resistance of *S. aureus* to meticillin constitutes a significant healthcare problem worldwide. MRSA is now thought to be responsible for 40% to 70% of *S. aureus* infections in intensive care units.[287] In the past decade new strains of MRSA have emerged in the community, causing infections in young, otherwise healthy people.[288] Meticillin resistance is determined by the *mecA* gene that lies on a novel genetic element in the genome of *S. aureus* known as the *SCCmec* cassette.[289] The gene product of *mecA* is a penicillin-binding protein (PBP2a) which renders *S. aureus* refractory to the action of all available β-lactam antimicrobials.[290] Because meticillin is rarely used today, the term "MRSA" is used now to describe strains of *S. aureus* resistant to all β-lactam antibiotics. Colonization with MRSA is more likely to result in infection than colonization with MSSA.[291, 292] MRSA has been isolated with increasing frequency from patients with bacterial keratitis.[293] It is a severe form of *S. aureus* keratitis and is being increasingly seen following keratorefractive surgery.[294] Community acquired meticillin *S. aureus* (CA-MRSA), in particular those associated with *lukSF-PV,* is present in epidemic proportions in the USA.[295] In the 2000s, the USA300 *S. aureus* strain was identified as the most common isolate responsible for outbreaks of CA-MRSA infections in the USA.[296] Although USA300 isolates were initially resistant only to β-lactam antimicrobials (mediated by *mecA*), they have broadened their resistance profiles considerably over the last 5 years. This includes resistance to clindamycin, tetracycline, vancomycin, gentamicin and the fluoroquinolones.

S. aureus resistance to fluoroquinolones emerged soon after their introduction in 1991. This was especially noted in MRSA strains.[297] Goldstein et al[263] examined the resistance of *S. aureus* isolates taken from patients with keratitis. They found that resistance significantly increased annually for ciprofloxacin from 6% in 1993 to 35% in 1997 and for ofloxacin from 5% to 35% over the same period. The fourth-generation fluoroquinolones have also not been a treatment panacea because of the emergence of resistance. Moshirfar et al[265] recently reported two cases of *S. aureus* associated keratitis after refractive corneal surgery that were resistant to the fourth generation moxifloxacin and gatifloxacin. *S. aureus* resistance to the fluoroquinolones is attributed to mutations that cause amino acid changes in one or both enzymes critical for bacterial DNA replication; DNA gyrase and Topoisomerase IV. This subsequently leads to reduced drug binding and efficacy.[298] Resistance is conferred by point mutations occurring in the subunits of *grlA* of topoisomerase IV and *gyrB* of DNA gyrase.[299] In some strains, overexpression of an efflux pump, termed NorA, contributes to the resistance phenotype.[300] Multiple mutations and combination of resistance mechanisms also confer cross-resistance to newer fluoroquinolones.[298]

5.132 P. aeruginosa resistance

P. aeruginosa is an extremely adaptable bacteria and is more than capable of developing resistance, in particular when antimicrobials are used extensively. Chaudhary et al[301] (Florida, USA) reported that the rate of ciprofloxacin resistance of *P. aeruginosa* taken from patients with keratitis rose significantly from 0.4% in 1991–1994 to 4% in 1995–1998. Garg et al[264] noted that 22 out of 141 cases of keratitis associated with *P. aeruginosa* were identified as resistant, using systemic breakpoints.

The three basic mechanisms of *P. aeruginosa* resistance; restricted uptake through its cell wall, drug efflux and drug inactivation will be discussed. Antimicrobial resistance through bacterial adherence to biofilm formation in contact lens patients has been mentioned previously (chapter 1.334).

P. aeruginosa has innate antimicrobial resistance due to the low permeability of antimicrobials through its cell wall. The outer membrane of *P. aeruginosa* presents a significant barrier to antimicrobials, restricting the rate of penetration of small hydrophilic molecules and excluding larger molecules. Small hydrophilic molecules such as β-lactams and fluoroquinolones must pass through aqueous channels provided by porin proteins.[302] The aminoglycosides do not pass through porin channels but bind to lipopolysacharide on the outer surface of the membrane, followed by active transportation into the cell.

Many classes of drugs are susceptible to multidrug efflux systems in *P. aeruginosa.*[303] The efflux system is composed of three protein components, an energy dependent pump, an outer membrane porin and a linker protein. Three main antimicrobial efflux systems have been described in *P. aeruginosa*: *mexAB-oprM*, responsible for the extrusion of β-lactams and fluoroquinolones; *mexXY-oprM,* responsible for the extrusion of the aminoglycosides, and *mexCD-oprJ* responsible for the extrusion of carbapenems and quinolones.[304]

P. aeruginosa are able to cause resistance by drug inactivation. Over-expression of the gene *ampC*, for example, leads to the production of β lactamase. This poses a threat to cephalosporins and other β lactam antimicrobials.[302] Aminoglycosides can be inactivated by the production of enzymes which transfer acetyl, phosphate or adnylyl groups on the antimicrobials.

Finally, *P. aeruginosa* can cause mutational changes in target enzymes, which result in maintaining their role in cell metabolism whilst causing resistance to certain antimicrobials. This mechanisms is most commonly seen with the fluoroquinolones through mutation in *gyrA*, a gene encoding the A subunit of the target enzyme DNA gyrase.[305]

5.2: Chapter aims

This chapter aims to compare the MICs of existing, recently introduced and potentially novel antimicrobials against bacterial isolates taken from patients with keratitis over two time periods. The *in vitro* interaction of clinically relevant antimicrobial combinations will also be described against a selection of isolates.

Pharmacokinetic properties of meropenem as a potential novel antimicrobial for the treatment of bacterial keratitis, will be investigated. Firstly the toxicity profile of meropenem will be examined using corneal cells in culture and secondly the corneal penetration of meropenem will be observed across donor human corneas mounted on artificial anterior chambers.

5.3 Methods

5.31 Minimum Inhibitory Concentration

Isolates were collected from patients with bacterial keratitis by the MOG in two time periods 2003-2005 and 2010-2011. Isolates were identified using methods described in chapter 2.12. MICs were calculated by E-Tests (chapter 2.21) for meropenem, moxifloxacin, gentamicin and ciprofloxacin against *P. aeruginosa*, and *Enterobacteriaceae*; and moxifloxacin, linezolid, ciprofloxacin, penicillin,

meropenem and teicoplanin against *S. aureus*. Mean MIC and MIC_{90} were calculated for each time period. An isolate was characterized as resistant if the MIC was greater than the systemic breakpoint MIC defined by BSAC (table 15).[118] It should be noted that breakpoints for the topical use of antimicrobials to treat bacterial keratitis are not available and may differ significantly from the systemic breakpoints.

Table 15: Antimicrobial resistance levels determined by the British Society of Antimicrobial Chemotherapy for *S. aureus*, *P. aeruginosa* and *S. pneumoniae*. Values determined from systemic data.

Antimicrobial	**Systemic breakpoint by BSAC (mg/L)**		
	S. aureus	*P. aeruginosa*	*S. pneumoniae*
Penicillin	>0.12	N/A	>2
Cefuroxime	N/A	N/A	>1
Chloramphenicol	>8	N/A	N/A
Gentamicin	>1	>4	>128
Vancomycin	>2	N/A	>2
Teicoplanin	>2	N/A	>2
Ciprofloxacin	>1	>1	>2
Moxifloxacin	>1	N/A	>0.5
Meropenem	N/A	>8	2

N/A: resistance values not available

5.32 Antimicrobial combinations

10 *S. aureus* and 10 *P. aeruginosa* isolates collected by the MOG from patients with bacterial keratitis were selected to investigate combinations of antimicrobials. Bacterial suspensions were prepared from colonies grown

overnight on agar plates, (chapter 2.2) 10 µL of which was evenly spread onto fresh agar plates. E-Test strips of two antimicrobials were placed on the plate forming a cross with a 90 degree angle. The intersection point of the cross was made to coincide with the MIC for each antimicrobial, calculated from previous experiments (figure 20). The plates were incubated at 35-37 °C in air for 18 hours, and the MIC of each antimicrobial in the combination was read. Each bacterial isolate was tested three times for each antimicrobial combination. The antimicrobial combinations tested for *S. aureus* were: teicoplanin and moxifloxacin, teicoplanin and ciprofloxacin, teicoplanin and meropenem, meropenem and linezolid, moxifloxacin and linezolid, meropenem and ciprofloxacin, and moxifloxacin and meropenem. The antimicrobial combinations tested for *P. aeruginosa* were: meropenem and ciprofloxacin, gentamicin and moxifloxacin, moxifloxacin and meropenem, meropenem and levofloxacin and gentamicin and ciprofloxacin.

Using the results of MICs determined with the antimicrobial alone and in combination, the fractional inhibitory concentration (FIC) was calculated, as per the following formula:-

$$\text{FIC of drug A} = \frac{\text{MIC drug A when tested in combination with drug B}}{\text{MIC of drug A alone}}$$

$$\text{FIC of drug B} = \frac{\text{MIC drug B when tested in combination with drug A}}{\text{MIC of drug B alone}}$$

$$\text{FIC} = \text{FIC drug A} + \text{FIC drug B}$$

The mean FIC of triplicate experiments for each antimicrobial combination for a particular isolate was then calculated. The mean FIC allowed the combination to be classified into one of 4 groups as defined by Pillai et al[306]; ≤ 0.5 = synergy, 0.5-1 = additivity, 1-4 = indifference and >4 = antagonism. For

example, Figure 21a shows synergy i.e. MICs of A and B are 1 mg/L and 0.5 mg/L, and decrease to 0.125 mg/L and 0.063 mg/L when measured in combination $FIC = \frac{0.125}{1} + \frac{0.063}{0.5} = 0.25$

Figure 21b shows additivity i.e. MICs of A and B are 1 mg/L and 0.5 mg/L, and decrease to 0.5 mg/L and 0.125 mg/L when measured in combination $FIC = \frac{0.5}{1} + \frac{0.125}{0.5} = 0.62$. It is apparent that a synergistic or additive effect can only occur for the combination if both FIC drug A and FIC drug B are each less than 1. Figure 21c shows indifference i.e. MICs of A and B are 1 mg/L and 0.5 mg/L with no change when measured in combination $FIC = \frac{1}{1} + \frac{0.5}{0.5} = 2$. Figure 21d demonstrates antagonism i.e. MICs of A and B are 1 mg/L and 0.5 mg/L, and increase to 8 mg/L and 4 mg/L after combination $FIC = \frac{8}{1} + \frac{4}{0.5} = 16$.

The mean, standard deviation (SD), minimum, maximum and coefficient of variance of the FIC was calculated for each antimicrobial combination against *S. aureus* and *P. aeruginosa* isolates.

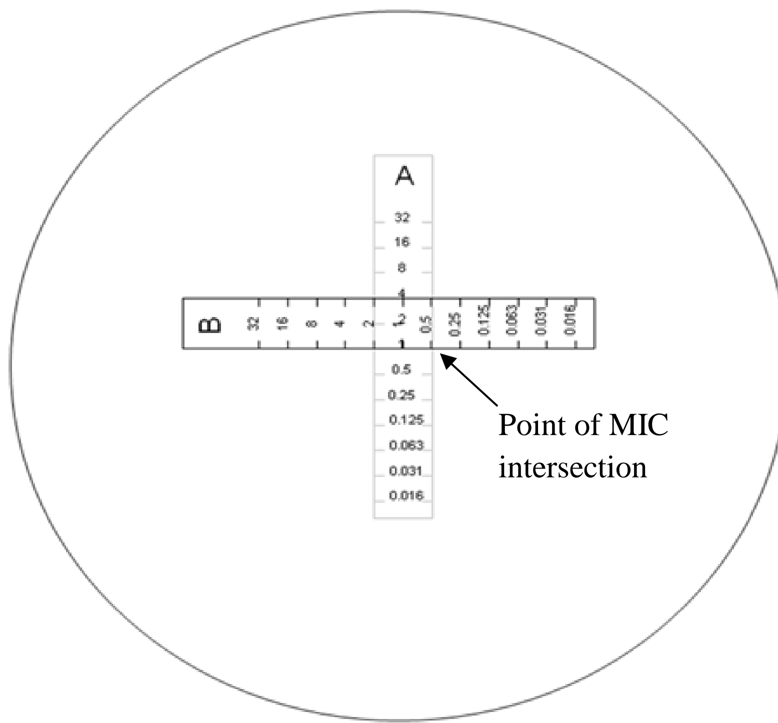


Figure 20. Illustration demonstrating the set up for E-Test combination testing of antimicrobial A and B (indicated at top of each E-Test) on an agar plate inoculated with bacteria. The two E-Tests are crossed at 90 degrees at the point of their individual minimum inhibitory concentrations i.e. MIC of A = 1 mg/L and B = 0.5mg/L.

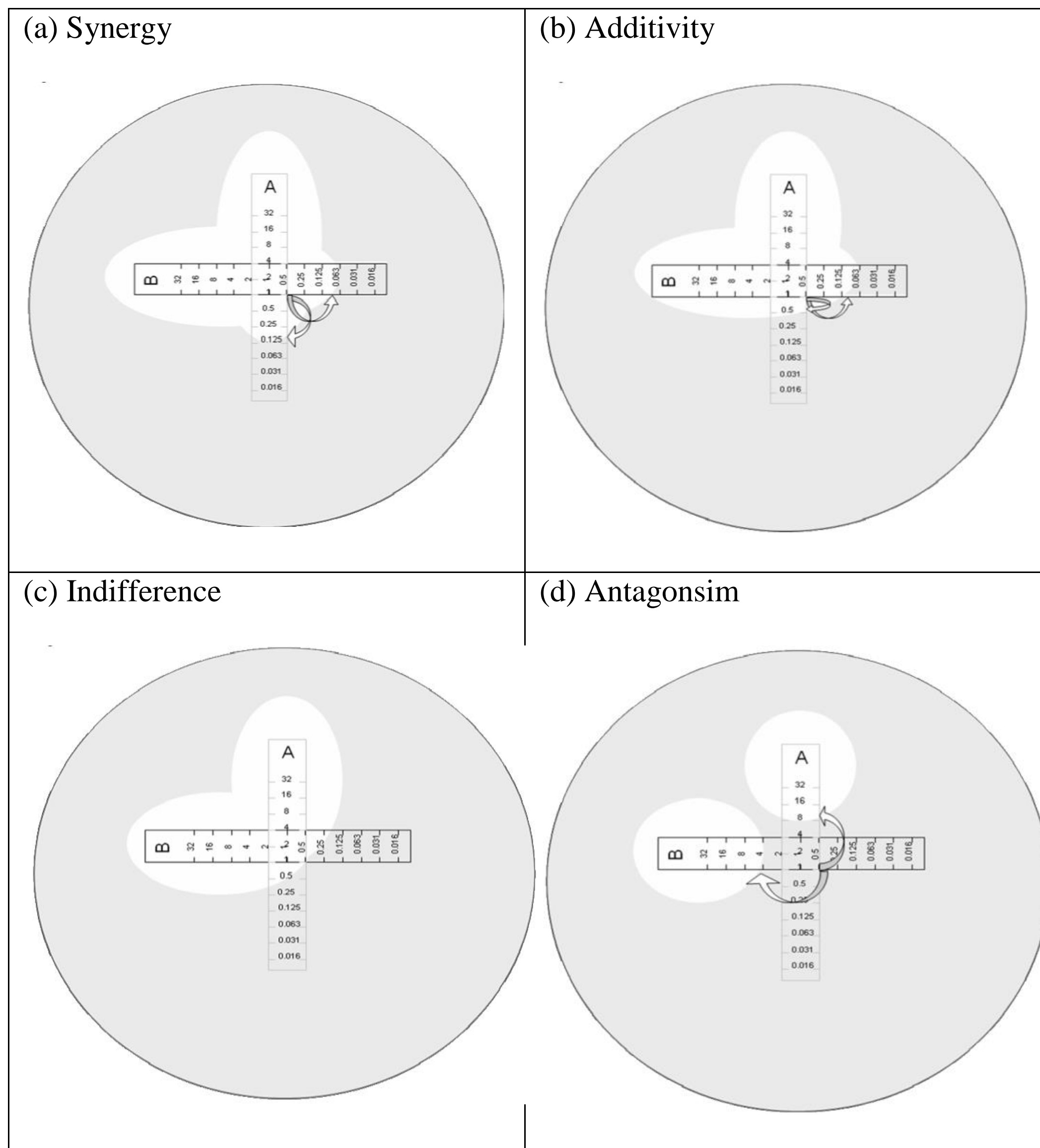


Figure 21. Illustrations demonstrating the appearance of E-Test combination experiments of antimicrobial A and B which result in (a) synergy, (b) additivity, (c) indifference and (d) antagonism.

5.33 Pharmacokinetics of meropenem

5.331 *Toxicity assays*

Meropenem was applied to HKs HCEs in culture, to investigate its cellular toxicity. Cell culture conditions were as described in chapter 2.4

5.3311 Methyl thiazolyl tetrazolium assay

Background: The methyl thiazolyl tetrazolium (MTT) assay, described by Mosman,[307] is a measure of mitochondrial cell function and hence is an indirect indicator of cell viability. It consists of the addition of the tetrazolium salt MTT which is reduced to a formazan product when added to growing cells. The resulting yellow to purple colour change is quantifiable on a 96 well plate reader.
Methods: The Cell Titer 96® Cell Proliferation Assay Kit (Promega, Southampton, United Kingdom) was used. HCEs and HKs were seeded at 5,000 cells per well in 96-well plates. Once confluence was achieved, cells were kept overnight in DMEM/F12 media without any supplements. Cells were treated with 5 mg/ml and 2.5 mg/ml of meropenem or moxifloxacin for 1 hour. 15 µl MTT dye solution was then added followed by incubation at 37 °C for 4 hours and 100 µl Stop Solution. The plates were then kept at 4 °C overnight in a moistened chamber. Each experiment was done in triplicate and blank controls (culture medium only), positive controls (cells plus medium) and negative controls (cells treated with the cytotoxin dimethyl sulfoxide (DMSO)) were included. Absorbance was read at 570 nm using an automated microplate reader (Bio-Rad, Hemel Hempstead, United Kingdom) and cell viability was expressed as percentages in relation to untreated controls. Values were expressed as the mean percentages of control values ± standard deviation (SD) from three independent

experiments. The Mann-Whitney U test (SPSS version 21) was used to compare cell viability between the drugs tested. A P-value <0.05 was considered to be statistically significant. Figure 22 shows the set-up of the 96 well-plates.

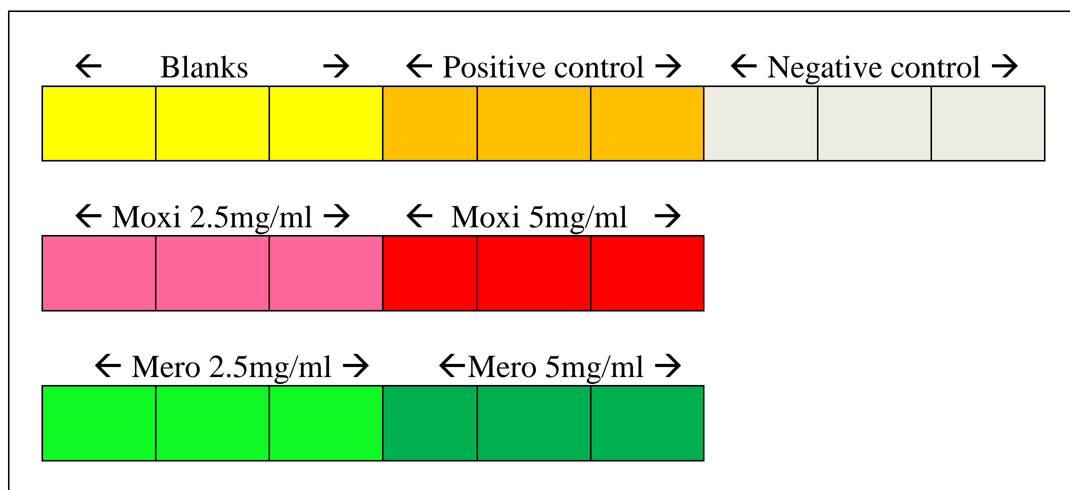


Figure 22: Illustration showing set-up of 96 well-plate for MTT assay of meropenem and moxifloxacin 2.5 mg/ml and 5 mg/ml, including positive and negative controls. Mero = meropenem and Moxi=moxifloxacin.

5.3312 Live dead assay

Background: Live Dead assay distinguishes live cells by the presence of intracellular esterase activity. This is determined by the enzymatic conversion of the non-fluorescent calcein acetomethoxy (AM) to the intensely fluorescent calcein. Calcein is well retained within live cells, producing an intense uniform green fluorescence (exitation/emmission 495 nm/515 nm). Conversely, ethidium enters cells with damaged cell membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells.

Methods: 24 well plates were seeded with 50,000 HKs or HCETs per well. Once confluence was achieved the cells were incubated overnight in DMEM/F12 media without any supplements. Each experiment included cells treated with meropenem 10 mg/ml for 1 hour, positive controls (cells plus medium) and negative controls (cells treated with DMSO). Three wells were used for each parameter and the experiment was repeated on three separate plates. 100 μl of a Live Dead assay (Invitrogen, Paisley, UK) containing 2 μM calcein AM and 4 μM ethidium homodimer-1 was added to each well and incubated at 37 ^{o}C in CO_2 for 1 hour. Cells were examined with an Axiovert 200 fluorescent microscope (Carl Zeiss). Green and red filters were used to photograph live and dead cells respectively. The number of cells was determined using an in house automated programme written in Matlab R14 (The Mathworks Inc., Natick, MA). In brief, the original image was pre-processed by an adaptive thresholding technique to improve the contrast and uneven illumination. A selective enhancement filter, initially described by Li et al,[308] was used to enhance the cells in the pre-processed image. Regions of interest (ROIs) were detected by thresholding the enhanced image and further refined by removing smaller objects. The number of cells was determined by labelling the remaining ROIs in the image.

5.332 Corneal and aqueous model

Human cadaver corneo-scleral discs stored in organ culture medium and not suitable for transplantation due to low endothelial cell density (ECD) <2200 cell / mm^2, were provided by the Manchester eye bank. Due to the reduced ECD and often absent or disrupted epithelium, and in order to make the corneas as similar as possible, the epithelial and endothelial layers were removed under the

dissecting microscope using cellulose eye spears (Beaver-Visitec, Oxfordshire, UK). Following trephination of a 9 mm central corneal disc, each cornea was washed in phosphate buffered saline (PBS) and mounted onto a flow type Franz Cell (PermeGear, Hellertown, USA) containing 5 ml PBS, at room temperature (figure 23). 50 µl of 10 mg/ml (500 µg) solution of meropenem was placed into the receptacle above the cornea. Prior to sampling, to ensure even mixing of meropenem in the artificial anterior chamber, fluid was repeatedly aspirated and injected via the sampling port, using a needle attached to a 20 ml syringe. 20 µl samples were collected from the artificial anterior chamber at 45 minutes, 1.5 hours, 4 hours and 24 hours and stored the corneas at -20 °C prior to processing. After 24 hours the corneas were washed and homogenised in 2 ml of PBS, centrifuged at 10,000 g for 5 minutes, and the supernatant transferred into a microtube. To ensure that the intra-stromal meropenem had been recovered from the corneas, three pellets were sequentially re-homogenised after reconstitutions in 2 ml PBS. Concentrations of meropenem were determined from the aqueous and corneal homogenates using a bioassay and high performance liquid chromatography (HPLC).

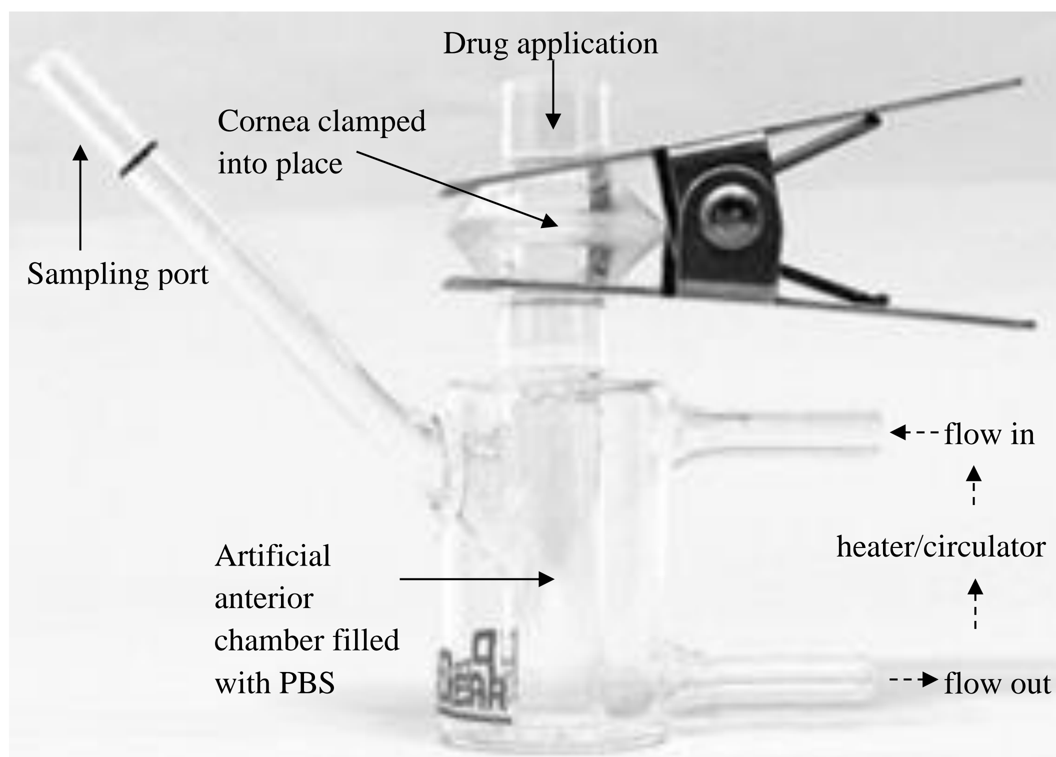


Figure 23**.** Franz Cell (PermeGear, US) used in meropenem penetration experiments. Flow in/flow out ports not used in this study.

5.3321 Bioassay

A disc diffusion bioassay was performed to estimate the concentration of meropenem in corneal homogenate and the anterior chamber samples. A standard curve was constructed using known concentrations of meropenem as follows. *E. coli* (ATCC 25922) was seeded on agar plates supplemented with horse blood and incubated overnight at 37 °C in air. Bacterial colonies were removed from the plates and added to sterile water to a 0.5 McFarland turbidity standard. The suspension was diluted 1 in 100 in sterile water, 10 µl of which was spread evenly onto fresh agar plates. Blank antimicrobial sensitivity discs were inoculated with 10 µl of meropenem and the plates incubated overnight in air at 37 °C. The zone of inhibition (ZOI) was calculated by measuring to the nearest millimetre the

diameter of the circle surrounding the disk devoid of bacterial growth (figure 24). Experiments were performed in triplicate for each of the 16 concentrations of meropenem from 0.01 to 313 μg/ml. A standard curve was constructed using a logarithm transformation of the data. To estimate the meropenem concentration in the anterior chamber fluid and corneal homogenate; 10 μl of the sample in question was placed onto blank discs on agar plates inoculated with *E. coli*. Plates were incubated overnight and ZOI were measured, as described above. Positive and negative controls were run in parallel with the test specimen.

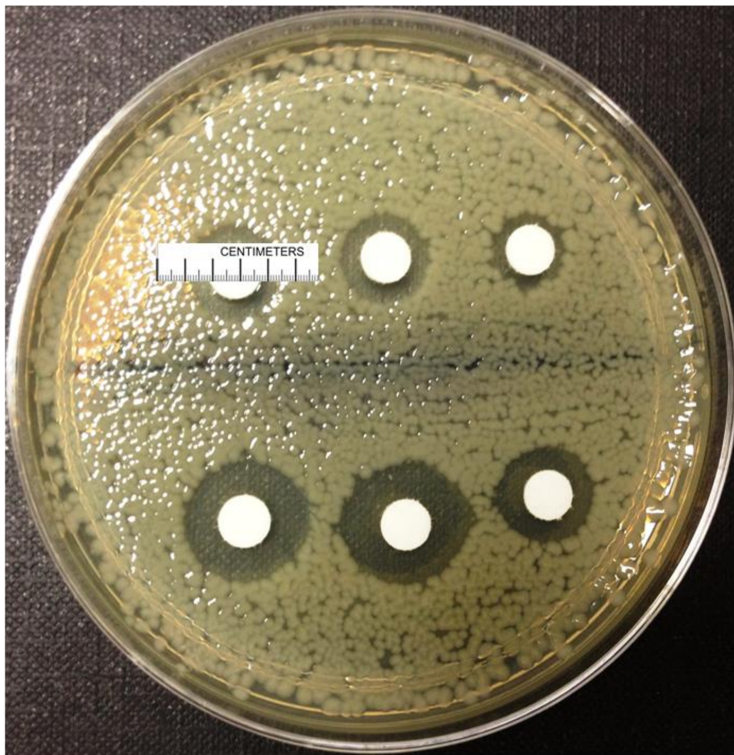


Figure 24. Example of meropenem bioassay. Discs containing various concentrations of meropenem placed onto agar plate inoculated with *E. coli.* Zone of inhibition (ZOI) surrounding disc after 24 hours incubation is measured to the nearest mm. For example ZOI top left = 32 mm.

5.3322 HPLC

Quantitation of meropenem in experimental samples was carried out using an isocratic reverse-phase high performance liquid chromatography (RP-HPLC) based on a method by Mendez et al.[309] Samples were centrifuged at 10,000 g for 5 minutes and 20 µL applied to a Phenomenex Luna 3u C18 100 Å RP-HPLC column (150 x 2.00 mm) equilibrated in 30 mM sodium phosphate, 12% acetonitrile, pH 3.0. Meropenem was eluted isocratically in the same buffer and typically emerged with a 5-6 minute residence time. Two HPLC systems were used for the analyses: a Beckman System Gold HPLC system with elution monitored at 300 nm or an ESA HPLC system fitted with an auto sampler and electrochemical detector with the electrode set at 600 mV. A standard curve of 0 – 50 µg/L meropenem in Hanks BSS was used for calibration in both cases. For the analysis of meropenem metabolites generated during the experiments, a gradient RP-HPLC separation was used. Samples were applied to the same column as above but which was equilibrated in 0.1% trifluoroacetic acid (TFA). Separation was carried out using a 30 minute gradient of 0 – 60 % [v/v] acetonitrile in 0.1% TFA, using a flow rate of 0.2 ml/min. Elution was monitored at 220 nm.

5.4 Results

5.41 MICs of antimicrobials against keratitis isolates

Table 16 shows the MICs and percentage of isolates resistant to the antimicrobials tested. Figure 25 shows histograms of MIC_{90} against across the 2 time periods. Using systemic breakpoints, 1% and 2% of *P. aeruginosa* isolates were resistant to ciprofloxacin in the 2003-05 and 2010-2011 time periods. 4% of isolates were resistant to *Enterobacteriaceae* in 2003-05, although this was not observed in the

later time period. High levels of ciprofloxacin resistance (18% and 8%) were seen in *S. aureus* isolates. Lower resistance was seen with moxifloxacin in *S. aureus*, 2% in 2003-2005 and 0% in 2010-2011. Similar low resistance to moxifloxacin was seen with *P. aeruginosa.* High gentamicin MICs and resistance were seen in 2010-2011 against *Enterobacteriaceae*. The sample size was low, however, in this cohort; n=16. The novel antimicrobial meropenem had low MICs in all isolates in both time periods with only 1 resistant isolate throughout (*P. aeruginosa*). Linezolid had low MICs with no resistance when tested against *S. aureus.*

Table 16: Minimum inhibitory concentrations (MICs) and resistance (using systemic breakpoints of antimicrobials) against (a) *P. aeruginosa* (b) *Enterobacteriaceae* and (c) *S. aureus* across two time periods: 2003-2005 and 2010-2011. Number of isolates tested (n), mean MIC (mean), MIC_{90} and % of isolates resistant using systemic breakpoints (resistance). Systemic breakpoint from BSAC indicated where available.

16a: MICs against *P. aeruginosa* (mg/ml)

	Year	n	mean	MIC_{90}	resistance	systemic breakpoint
Ciprofloxacin	2003-2005	140	0.2	0.5	1%	>1
	2010-2011	51	1.4	0.2	2%	
Moxifloxacin	2003-2005	140	0.8	1.0	N/A	N/A
	2010-2011	51	0.7	1.0	N/A	
Gentamicin	2003-2005	140	3.4	2.0	4%	>4
	2010-2011	51	1.3	2.0	0%	
Meropenem	2003-2005	140	0.4	0.3	0%	>8
	2010-2011	51	0.2	0.4	1.9%	

16b. MICS against *Enterobacteriaceae* (mg/ml)

		n	mean	MIC_{90}	resistance	systemic breakpoint
Ciprofloxacin	2003-2005	84	0.1	0.1	4%	>1
	2010-2011	16	0.1	0.2	0%	
Moxifloxacin	2003-2005	84	0.1	0.3	0%	>1
	2010-2011	16	0.3	0.9	0%	
Gentamicin	2003-2005	84	1.1	1.0	1%	>4
	2010-2011	16	1.6	4.0	13%	
Meropenem	2003-2005	84	0.1	0.1	0%	>8
	2010-2011	16	0.03	0.05	0%	

16c. MICS against *S. aureus* (mg/ml)

		n	mean	MIC_{90}	resistance	systemic breakpoint
ciprofloxacin	2003-2005	94	5.0	32.0	18%	>1
	2010-2011	39	2.6	0.6	8%	
moxifloxacin	2003-2005	94	0.2	0.25	2%	>1
	2010-2011	39	0.1	0.1	0%	
Teicoplanin	2003-2005	94	1.5	0.9	0%	>2
	2010-2011	39	0.1	0.1	0%	
Penicillin	2003-2005	94	1.5	3.2	78%	>0.12
	2010-2011	38	0.4	0.8	66%	
Linezolid	2003-2005	94	0.8	0.5	0%	>4
	2010-2011	40	0.7	1.0	0%	
Meropenem	2003-2005	94	0.08	0.12	N/A	N/A

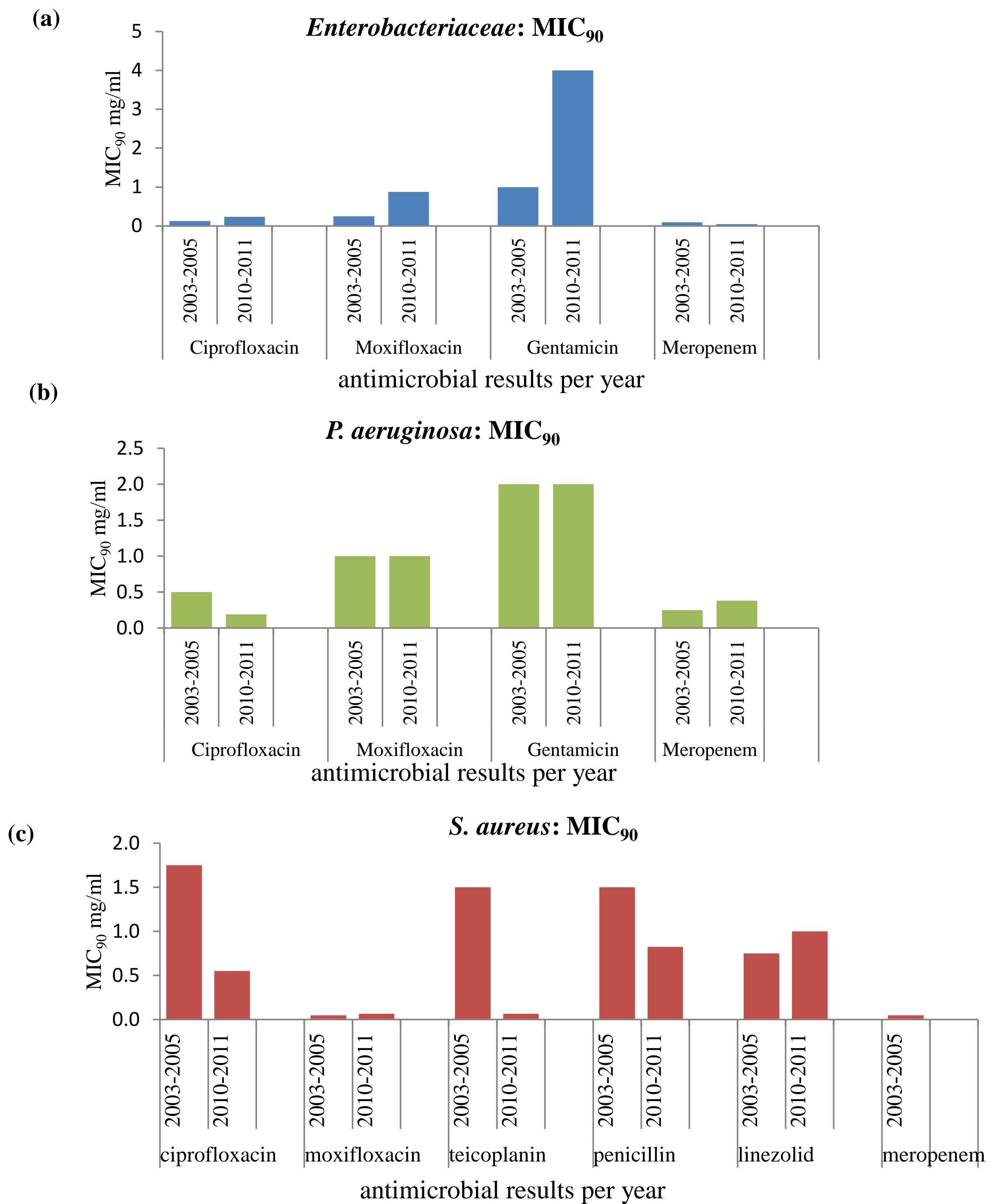


Figure 25: Histograms showing MIC_{90} of antimicrobials against (a) *P. aeruginosa*, (b) *Enterobacteriaceae* and (c) *S. aureus* across 2 time periods; 2003-05 and 2010-11.

5.42 in vitro combination of antimicrobials

The results for each antimicrobial combination against *S. aureus* and *P. aeruginosa* are shown in tables 17a and 17b. The coefficient of variance for the antimicrobial combinations was 11% against *S. aureus* and 22% against *P. aeruginosa*. The antimicrobial combinations with the greatest additive effect against *S. aureus* were meropenem and teicoplanin, meropenem and ciprofloxacin, and moxifloxacin and teicoplanin. Synergy was demonstrated in 20% of isolates with the combination of meropenem and teicoplanin and in 10% of isolates with meropenem and ciprofloxacin. Synergy or additivity was seen in 80% in each of those two combinations. The remaining combinations including ciprofloxacin and teicoplanin, linezolid and moxifloxacin, moxifloxacin and meropenem and linezolid and meropenem demonstrated a predominantly indifferent interaction. No antagonism was seen in any of the experiments. Of the combinations against *P. aeruginosa* isolates (table 17b), meropenem and ciprofloxacin demonstrated the lowest mean FIC (0.7) with synergy observed in 10% and additivity seen in 80%. The interaction between gentamicin with moxifloxacin, meropenem and moxifloxacin, meropenem and levofloxacin and gentamicin and ciprofloxacin were predominantly indifferent. No antagonistic effect was seen, although one isolate demonstrated a mean FIC of the three tests of 3.7 (3.5, 3.5 and 4.1) with the combination of meropenem and moxifloxacin.

Table 17. *in vitro* activity of antimicrobials individually and in combination against (a) 10 *S. aureus* isolates and (b) 10 *P. aeruginosa* isolates, determined by E-Test method. Mean minimum inhibitory concentrations (MICs) calculated for each antimicrobial alone and in combination with another antimicrobial (mean values indicated). Fractional inhibitory concentration (FIC) determined for each combination, mean FIC (SD) and FIC range indicated. FIC interpretation of combinations (% of isolates) synergistic (Syn) FIC≤ 0.5, additive (Add) FIC= 0.5-1, indifferent (Ind) FIC= 1-4, or antagonistic (Ant) FIC>4.

(a): MICs and FICs for antimicrobials against *S. aureus* isolates

	mean MIC (mg/l)		**FIC**		**FIC interpretation**			
	alone	**in combination**	**range**	**mean (SD)**	**Ant (%)**	**Ind (%)**	**Add (%)**	**Syn (%)**
meropenem & teicoplanin	0.4 1.0	0.1 0.3	0.3 - 1.3	0.8 (0.2)	0	20	60	20
meropenem & ciprofloxacin	0.4 16.5	0.2 9.8	0.3 - 2.0	0.9 (0.3)	0	20	70	10
meropenem & moxifloxacin	0.4 0.8	0.1 0.6	0.6 - 2.0	1.2 (0.2)	0	60	40	0
meropenem & linezolid	0.4 0.5	0.4 0.5	1.6 - 2.0	1.9 (0.1)	0	100	0	0
moxifloxacin & linezolid	0.8 0.5	0.8 0.5	1.8 - 2.0	2.0 (0.0)	0	100	0	0
moxifloxacin & teicoplanin	0.8 1.0	0.6 0.3	0.5 - 1.3	0.9 (0.1)	0	40	60	0
ciprofloxacin & teicoplanin	16.5 1.0	13 0.2	0.3 - 2.0	1.0 (0.1)	0	40	50	10

(b): MICs and FICs for antimicrobials against *P. aeruginosa* isolates

	mean MIC (mg/l)		FIC		FIC interpretation			
	alone	in combination	range	mean (SD)	Ant (%)	Ind (%)	Add (%)	Syn (%)
meropenem & ciprofloxacin	2.0 0.1	0.5 0.03	0.3-1.7	0.7 (0.2)	0	10	80	10
meropenem & moxifloxacin	2.0 2.5	0.7 0.5	0.4-3.7	1.3 (0.2)	0	50	30	20
meropenem & levofloxacin	2.0 0.7	0.7 0.4	0.5-2.0	1.8 (0.5)	10	80	10	0
gentamicin & ciprofloxacin	1.0 0.1	0.8 0.1	0.8-3.1	1.9 (0.4)	10	13	6	0
gentamicin & moxifloxacin	1.0 2.5	0.6 0.6	0.3-2.0	1.3 (0.2)	0	80	0	20

5.43 In vitro toxicity of meropenem and moxifloxacin

MTT assays of HCEs and HKs showed meropenem had significantly higher cell viability at both 5 mg/ml and 2.5 mg/ml compared to moxifloxacin (p=0.029 and p=0.018 respectively) (figure 26). Live dead assay of HCEs showed the viability of cells treated with meropenem was 96%, compared to cell 95% in the untreated control cells (p=0.52). The high cell viability in meropenem treated cells in both assays, suggests low cell toxicity.

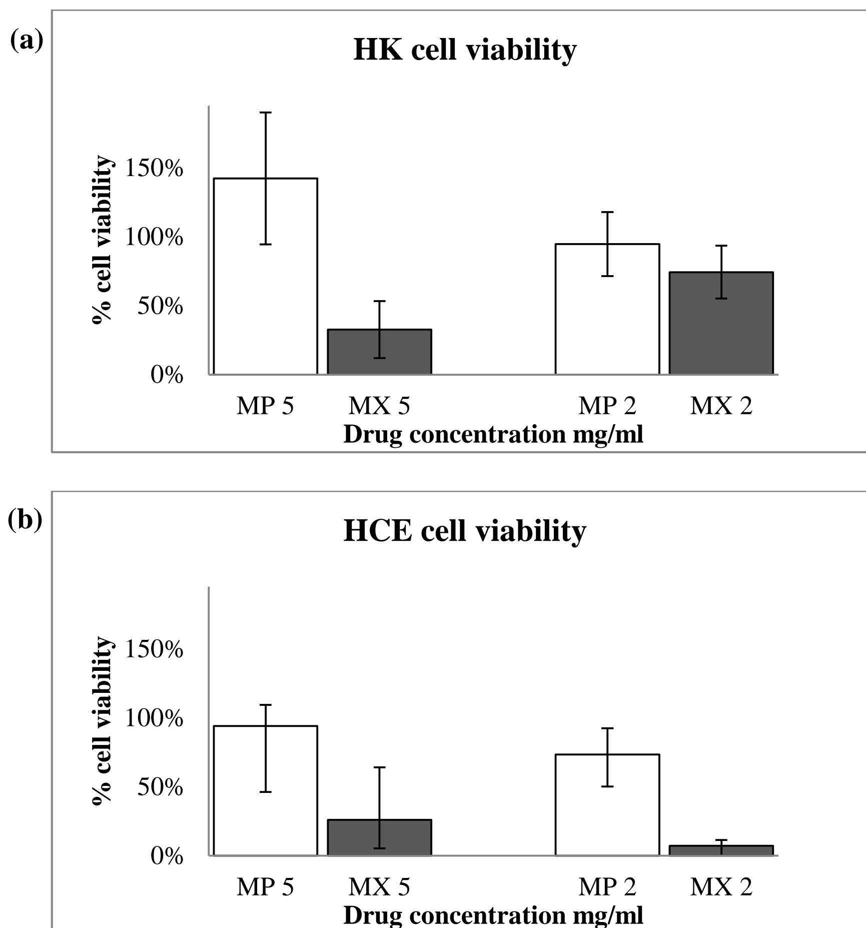


Figure 26. MTT cell viability assay of cultured (a) HKs and (b) HCEs incubated for 1hr with meropenem (MP) and moxifloxacin (MX) 5 and 2 mg/ml. Values are expressed as percentage of control (viability in absence of drug). Results are shown as the mean ± SD (n =6, $p < 0.01$).

5.44 ***Concentration of meropenem in chamber and corneal tissue***

The standard curve for the meropenem bioassay gave a coefficient of determination of R^2=0.99, $p<0.01$). Anterior chamber concentrations of meropenem taken at 45 minutes, were lower than the MIC_{90} of *E. coli* (0.094 μg/ml) in 33% (7/18) of corneas in the bioassay group and 17% (3/18) of corneas in the HPLC group. In all subsequent sampling points, however, anterior chamber meropenem concentrations exceeded the MIC_{90} of *E. coli*, using both HPLC and

bioassay. Variability was seen between the corneas in the aqueous meropenem concentration calculated by both bioassay and HPLC (figure 27).

The mean meropenem concentration in the anterior chamber at 45 minutes was 1.3 (±1.5) μg/ml and 0.9 (±0.9) μg/ml using the bioassay and HPLC, respectively (table 18). Aqueous concentrations increased steadily with time and at 24 hours, mean values were 43.7 (±27.2) μg/ml (bioassay), and 13.5 (±14.8) μg/ml (HPLC). At the 24 hour time point, meropenem concentration measured by bioassay exceeded the measurements made by HPLC in 12 out of the 18 corneas. Figure 28 demonstrates HPLC meropenem metabolite analysis. The presence of a single peak of meropenem can be seen in Figure 28a in a sample containing freshly made meropenem. The presences of other peaks are observed samples are analysed from later time points. The compartmentalisation of meropenem in the cornea and aqueous at 24 hours, is shown in Figure 29.

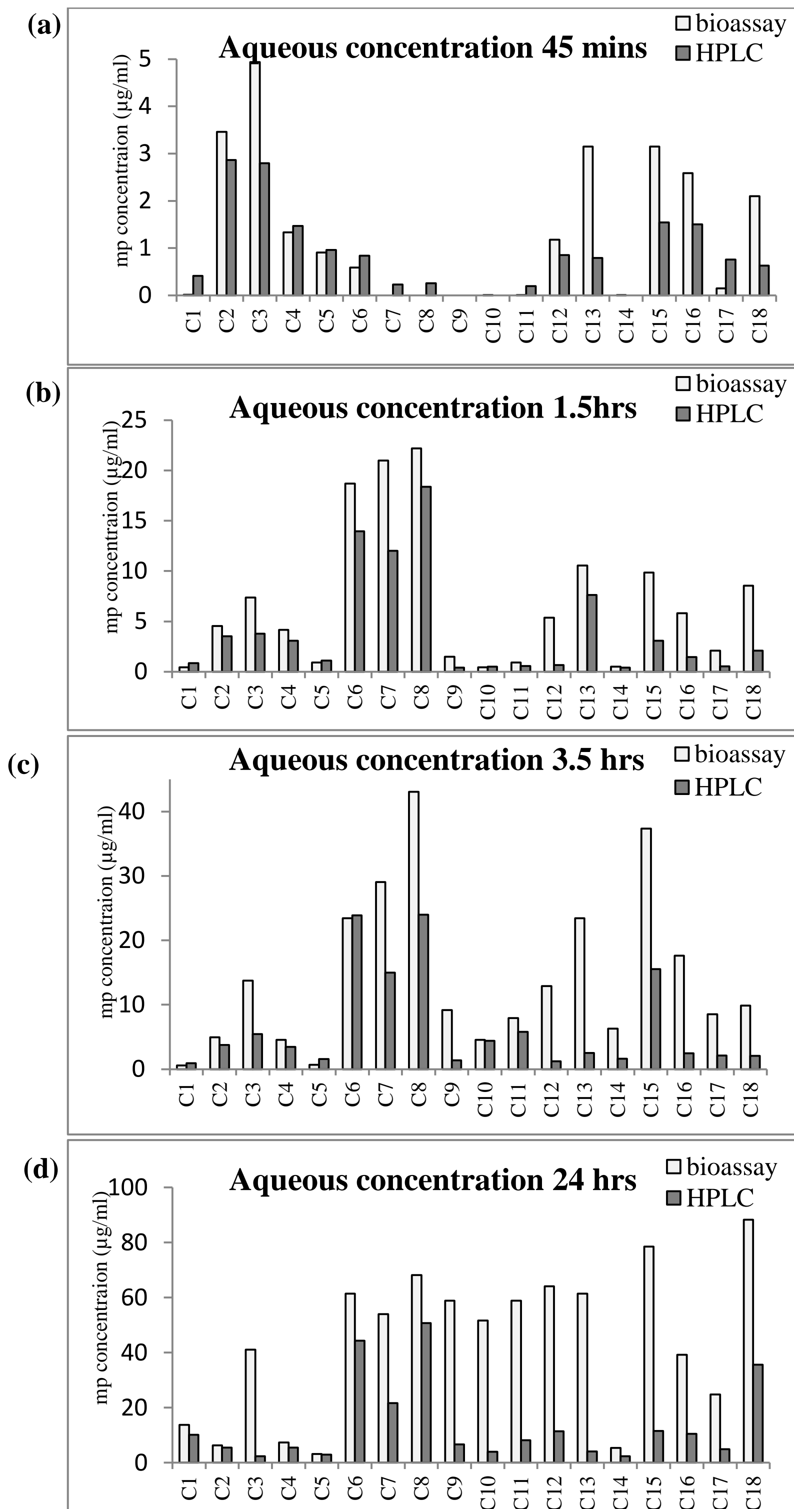


Figure 27. Aqueous concentration of meropenem determined after the application of meropenem 10 mg/ml on a donor human cornea across the artificial anterior chamber in 18 corneas (C1 to C18). Measurements made using bioassay and HPLC at (a) 45 minutes, (b) 1.5 hours, (c) 3.5 hours and (d) 24 hours.

Table 18. Mean concentration of meropenem (µg/ml) collected in Franz Cell artificial anterior chamber after application of 500 µg meropenem onto human corneas, n=18

	Bioassay (µg/ml)		**HPLC** (µg/ml)	
Sample time	**Mean**	**SD**	**Mean**	**SD**
45 min	1.3	1.5	0.9	0.9
1.5 hr	6.9	7.1	4.1	5.3
3.5 hr	14.3	12.4	6.5	7.6
24 hr	43.7	27.2	13.5	14.8

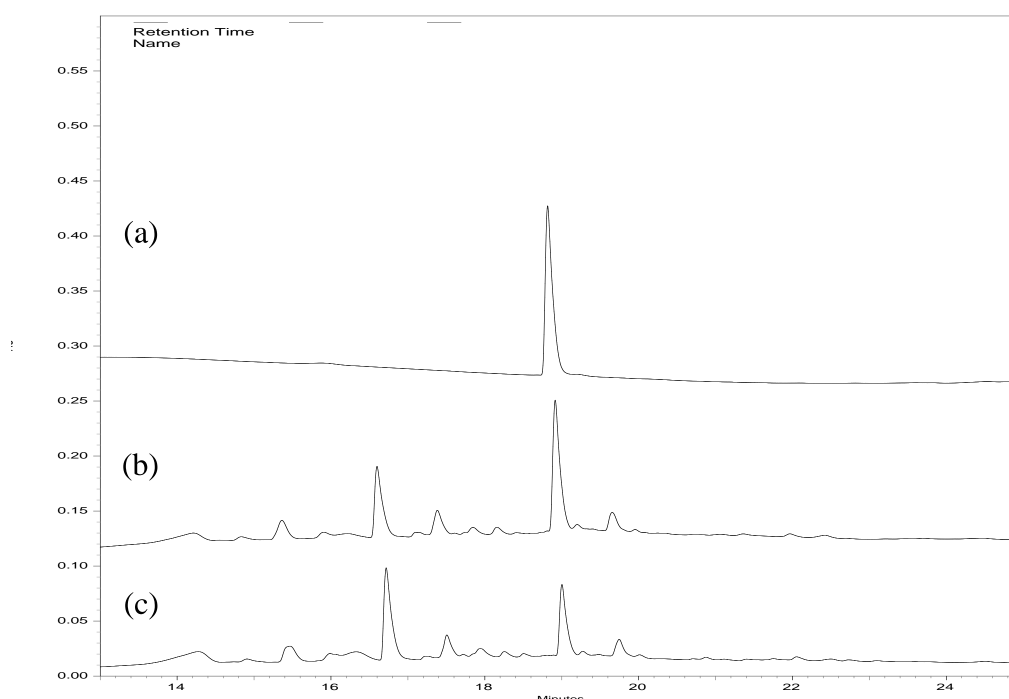


Figure 28. Detection of the presence of meropenem metabolites during penetration experiments, using RP-HPLC. Aqueous samples were analysed using a gradient RP-HPLC separation with elution monitored at 220 nm. (a) fresh meropenem sample (10 mg/ml), (b) cornea 5 aqueous sample at 3.5 hours and (c) cornea 5 aqueous sample at 24 hours.

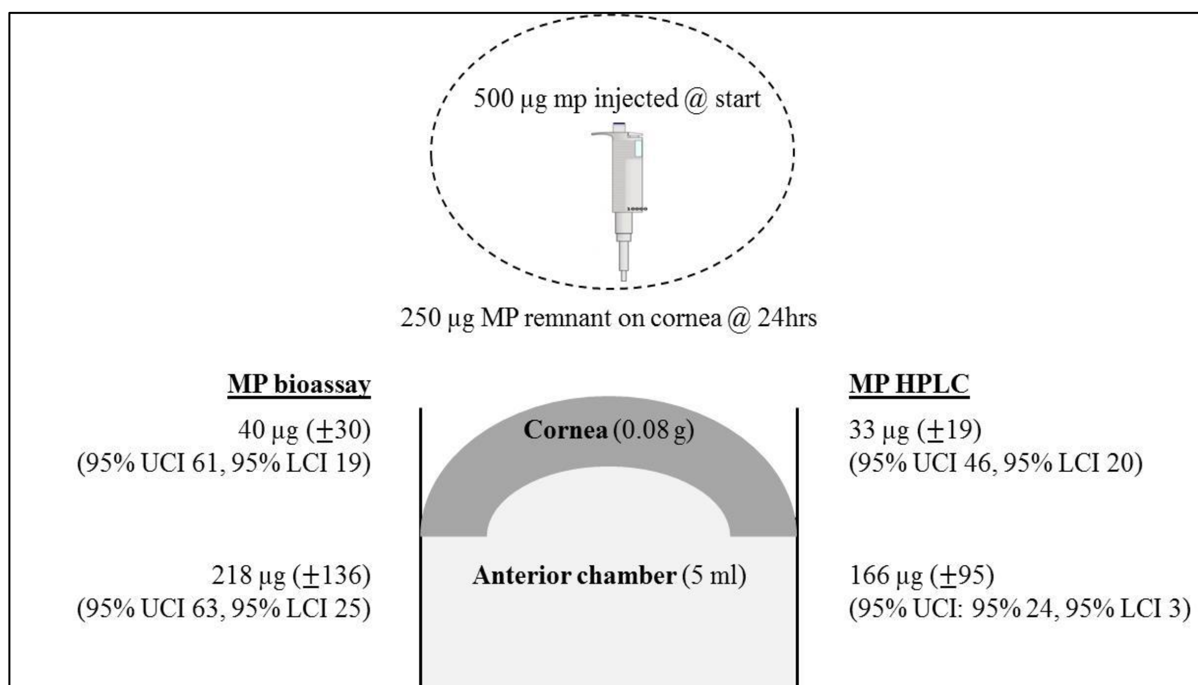


Figure 29. Compartmentalisation of meropenem (mp) at 24 hours in the cornea and aqueous, following the application of 500 µg meropenem, (n=18) onto human corneas set up in the in Franz Cell. Mean values of meropenem (µg), from bioassay and HPLC used. SD = standard deviation, UCI = upper confidence intervals, LCI = lower confidence intervals.

5.5 Discussion

5.51 Selecting an antimicrobial for further study based on in vitro study

Prompt and effective treatment of bacterial keratitis is critical in reducing the impact of this sight-threatening disease. There are several considerations when choosing the most appropriate antimicrobial therapy for bacterial keratitis. First, because an increase in bacterial resistance to the standard antimicrobials used to treat bacterial keratitis has been reported,[263, 264, 266, 310] it is essential to establish contemporaneous data on the spectrum of causative microorganisms and their

expected resistance profile.[104] Temporal changes in the spectrum of pathogens and resistance have also been noted worldwide (table 3) and monitoring the susceptibility of bacterial isolates to current antimicrobials and evaluation of novel therapies is therefore important. Second, the absence of established breakpoint concentrations for antimicrobials when they are used topically makes the interpretation of MIC data difficult. The susceptibility criteria used to select an antimicrobial for treatment are based on the anticipated response of the bacteria against concentrations of the antimicrobial that can be achieved in serum.[118, 311] Topical application of an antimicrobial to the cornea may achieve a very different concentration and bioavailability in the tissue than the serum levels.[312] Although the appropriate disc susceptibility breakpoint for each antimicrobial and bacterial isolate combination has not yet been determined, there is good evidence[4, 313] demonstrating the relationship between the MIC of topically applied antimicrobials and clinical outcome in bacterial keratitis. This relationship is particularly well established for pathogenic bacteria such as *P. aeruginosa* and *S. aureus*.[4] The MIC is therefore an important measure for evaluating the potential effectiveness of topically applied antimicrobials in the treatment of bacterial keratitis. In this study, systemic breakpoint standards from the BSAC were used for reference, although these may not be relevant for interpreting the sensitivity of ocular isolates.

Based on systemic breakpoints, this study found resistance in several commonly used antimicrobials across two time periods; 2003-05 and 2010-11. Particularly worrying was the high level of resistance in the commonly used ciprofloxacin against *S. aureus.* All the Gram-positive isolates tested were susceptible, however, to teicoplanin and the novel antimicrobials, meropenem,

and linezolid. Lower resistance was seen against the Gram-negatives tested. Overall, the *in vitro* data suggest that, of the antimicrobials currently in use, moxifloxacin offers the best coverage against both Gram-positive and Gram-negative isolates. Moxifloxacin has also been shown in other studies to have both good patient tolerability[314] and pharmacokinetics.[268] Of the newer antimicrobials potentially suitable for ophthalmic use, meropenem appears to be a good choice for empiric monotherapy in bacterial keratitis, offering broad-spectrum cover against both Gram-positive and Gram-negative microorganisms, with only one of the 772 isolates tested (*P. aeruginosa*) found to be resistant. Linezolid was active against all *S. aureus* isolates including a small number of MRSA isolates. Linezolid may therefore be useful for dual therapy when used in combination with an antimicrobial with good cover against Gram-negative bacteria.

5.52 Combination testing in vitro

As opposed to single therapy, an antimicrobial combination offers a broader spectrum of activity and may reduce selective pressures. In addition, combination therapy may also result in synergy (chapter 5.126). If a combination of antimicrobials demonstrates a synergistic or additive effect as determined by MIC this combination may prove more effective than monotherapy with the individual agents. It should be noted that the definitions of synergy through to antagonism[282, 315] are definitions that relate to interaction *in vitro* and it is unknown whether they translate into an improved outcome for topical combination therapy. If the extrapolation to an *in vivo* effect is valid, a synergistic or additive antimicrobial combination does offer a broader spectrum of activity[62] that may reduce selective pressures and the emergence of resistance. The traditional approaches to assess

antimicrobial combinations *in vitro* are by checkerboard and time kill methods.[316] These methods are however, time and material expensive and for that reason they are not used routine in routine clinical practice. The method used in this study with pairs of E-Test strips is relatively new and has the advantage that it is easy and cheap to perform.[317] The degree of agreement between FIC results calculated by the checkerboard and the E-Test method varies in the literature depending on the type bacteria tested. For example 55% agreement was found between the two tests when used with *Brucella melitensis* isolates,[315] 63% with *Acinetobacter*[318] and 90% with *P. aeruginosa*.[319] A limitation of the E-Test method is that it does not provide information about the bactericidal activity of the combination.

The current study showed that the E-Test method has a reasonably low coefficient of variance and is particularly useful to screen a large number of isolates against several combinations of antimicrobials. The combination of meropenem and teicoplanin gave the lowest mean FIC for *S. aureus*, with synergy or antagonism seen in 80%. For *P. aeruginosa* the combination with the lowest mean FIC was meropenem and ciprofloxacin with synergy or antagonism seen in 90%. Against *S. aureus* the combinations of teicoplanin with meropenem, ciprofloxacin or moxifloxacin also gave a low mean FIC with more than 50% demonstrating either an additive effect or synergy. Other combinations tested were predominantly indifferent. No combinations were found, of antimicrobials that were consistently antagonistic, when used against *S. aureus* or *P. aeruginosa*.

5.53 Pharmacokinetics of meropenem

Any conclusions about antimicrobial treatment of bacterial keratitis based on *in vitro* studies of drugs have inherent limitations. As previously discussed (chapter 5.112), the breakpoints for the antimicrobials are determined from expected serum

concentrations after systemic administration and breakpoints after topical administration have yet to be standardised. More information about the bioavailability of antimicrobials delivered topically to the cornea is needed to interpret the MIC levels. This is especially relevant to the novel antimicrobials tested. The antimicrobial meropenem was selected to undergo pharmacokinetic testing based on the *in vitro* results from these studies.

Topically applied drops are rapidly lost from the ocular surface and therefore bioavailability of topically applied drugs is very low, typically less than 5%.[252] In order to reach adequate tissue concentrations in the cornea, high drug concentrations coupled with frequent application are required. An assessment of corneal cellular toxicity is therefore an important aspect of analysing a drug that is to be applied to the eye. In this study we used the MTT assay[16] to assess the toxicity of meropenem. The MTT assay is a measure of mitochondrial cell function and hence is an indirect indicator of cell viability. Saarinen-Savolainen et al[320] have shown the MTT assay in immortalised corneal cell lines to be an accurate and reliable method to assess for the toxicity of topically applied drugs to the cornea. We found the cellular toxicity of meropenem on HKs and HCEs was significantly lower than moxifloxacin. The low cell viability we found in corneal cells treated with moxifloxacin was similar to previous studies.[321] The Live Dead assay distinguishes live cells by the presence of intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent calcein AM to the intensely fluorescent calcein. The Live Dead assay in this study showed similar high levels of cell viability in both meropenem treated and untreated (control) cells. The low level of toxicity, seen in both MTT and Live Dead assay, parallels the good systemic safety profile of meropenem.[322, 278, 323]

The study of antimicrobial corneal pharmacokinetics is commonly performed in animals such as rabbits.[324, 325] There are several structural, physiological and biochemical differences between the human and the rabbit eye.[326] Rabbits have a nictitating membrane, a larger corneal surface area and a thinner cornea, all of which could alter corneal pharmacokinetics. Studying corneal pharmacokinetics *in vivo* in human subjects is ideal but this is a long, costly process. The method used in this study of testing corneal penetration with *ex-vivo* human corneas on artificial anterior chambers, allows testing of human tissue without the constraints of testing on live patients.

Two methods of analysing meropenem concentration were used in this study; HPLC and bioassay. Chemical assays are sensitive but may not reflect the activity of the compound particularly in the tissue which may be affected for example, by pH and protein binding.[327, 328] A further important difference in considering the data between both methods relates to their ability to measure active metabolites. HPLC detects compounds with predefined chemical structures and (not necessarily) different chemical species that result from metabolic reactions. The extra peaks on the HPLC traces, seen in the older aqueous samples (figure 28), may therefore represent metabolites of meropenem. A bioassay will detect any active substances against the test bacteria, irrespective of their chemical characteristics.[329] The higher concentrations using the bioassay, therefore, may suggest the metabolites of meropenem are biologically active, which could explain the higher meropenem concentration calculated in bioassay, compared to HPLC. Further studies to investigate the metabolic products of meropenem would be indicated.

An important factor that determines the efficacy of an antimicrobial is its ability to penetrate to the target ocular tissues at concentrations greater than the MIC. Sueke et al[104] calculated the MIC_{90} of meropenem to be 0.08 µg/ml to *S. aureus*; 0.25 µg/ml to *P. aeruginosa*; and 0.054 µg/ml to Streptococci against isolates from patients with keratitis. The mean concentration of meropenem in the aqueous of the artificial chamber detected by both HPLC and bioassay methods superseded the MIC_{90}, at even the earliest (45 minute) sampling point. To achieve standardisation of the corneas, the remaining epithelium and endothelium was removed. An ulcer is usually present in most patients with bacterial keratitis and would support this *in vitro* model. It is unclear however, whether the presence of an intact functioning endothelium would affect the pharmacokinetics of meropenem across the cornea into the anterior chamber, and therefore further work using *in vivo* studies would be appropriate. Whether the same concentrations would be achieved *in vivo* is unclear but these results would support the consideration of meropenem in the treatment of bacterial keratitis.

5.6 Conclusion

The antimicrobial meropenem was found to have excellent pharmacodynamic properties, with low MICs against a wide range of isolates from patients with bacterial keratitis, both singly and in combination with other antimicrobials. Further pharmacokinetic studies, showed excellent penetration of meropenem in an artificial anterior chamber model and low cellular toxicity. Prior to initiating meropenem as a treatment in bacterial keratitis further pharmacokinetic and safety data should be obtained from live human subjects.

CHAPTER 6: SUMMARY AND FURTHER WORK

6.1 Summary of *S. aureus* study (chapter 3)

The prevalence, genetic diversity and clinical relevance of the *lukSF-PV* gene, encoding the bacterial toxin PVL, were investigated in *S. aureus*, isolated from cases of bacterial keratitis in the UK. Multiplex PCR investigating carriage of *lukSF-PV* and *mecA* were performed on 95 *S. aureus* isolates taken from patients with keratitis. The *lukSF-PV* operon was sequenced to investigate its diversity and MLST to test for a clonal relationship between *lukSF-PV* isolates. Antimicrobial MICs and clinical outcome data were compared for isolates characterized as *lukSF-PV*+ve, *mecA*+ve, and *lukSF-PV/mecA*-ve.

Out of 95 isolates, 9 (9.5%) were *lukSF-PV*+ve, 9 (9.5%) *mecA*+ve and 1 was positive for both. Five SNPs were found in *lukSF-PV* genes of 7 strains. There was no significant difference between the MICs of *lukSF-PV/mecA*-ve and *lukSF-PV*+ve isolates to the antimicrobials tested, except for tigecycline ($p<0.05$). The *mecA*+ve isolates had significantly higher mean MICs to meropenem and fluoroquinolones ($p<0.05$). There were non-significant trends for healing and treatment times, ulcer and scar size and overall clinical score to be greater in the *lukSF-PV*+ve group. The proportion of patients, however, that required surgery was significantly greater amongst patients with *lukSF-PV*+ve isolates with an odds ratio of 7.8 (95% CI 1-42, $p=0.018$) for patients requiring surgery.

In conclusion; patients with *lukSF-PV*+ve *S. aureus* were associated with a trend to worse clinical outcome and more surgical interventions, with an effect unrelated to MICs. This suggests that *lukSF-PV* may be an important virulence

factor in *S. aureus* associated keratitis. To further ascertain the relevance of *lukSF-PV* in *S. aureus* keratitis, a larger study would be needed. This is especially the case in light of previous epidemiological studies that suggest that the presence of PVL is not associated with a worse clinical outcome in *S. aureus* infection in other organs.

6.2 Summary of *P. aeruginosa* study (Chapter 4)

To examine temporal dynamics of keratitis-associated *P. aeruginosa*, the genetic characteristics of isolates collected during 2003-04 published by Stewart et al[231] and 2009-10 were compared using an AT genotyping system.

60 *P. aeruginosa* isolates were collected by the MOG from patients with keratitis from 2009 to 2010. Results from AT genotyping were compared to similar isolates collected by the MOG between 2003 and 2004. The distribution of keratitis-associated isolates from the two time periods (n=123) among a database of *P. aeruginosa* strains of non-ocular origin (n=322) indicated that 71% of UK keratitis-associated *P. aeruginosa* isolates clustered together. There was no evidence for major variations in the distribution of clone types between the two collections. The "core keratitis cluster" is related to the *P. aeruginosa* eccB clonal complex, which is associated with adaptation to survival in environmental water. This suggests that adaptation to environmental water is a key factor in the ability of *P. aeruginosa* to cause eye infections which is consistent with the notion that aquatic environments are integral to the transmission dynamics of *P. aeruginosa*, in the context of bacterial keratitis. However, the link between specific genotypes and clinical outcome or risk factors is not clear.

Further analysis of clinical data and studies involving additional sets of patients for verification of this hypothesis will provide a clearer picture, helping to

link genetic features with evidence-led clinical management of *P. aeruginosa* keratitis.

6.3 Summary of antimicrobial study (chapter 5)

6.31 Pharmacodynamics of single and combinations of antimicrobials

Pharmacodynamic properties of commonly used and potentially novel antimicrobials were determined by calculating MICs against isolates from patients with keratitis in 2003-2005 and 2010-2011. MICs were calculated by E-Tests for meropenem, moxifloxacin, gentamicin and ciprofloxacin against *P. aeruginosa*, and *Enterobacteriaceae* and moxifloxacin, linezolid, ciprofloxacin, penicillin, meropenem and teicoplanin against *S. aureus*. Based on systemic breakpoints this study found resistance in several commonly used antimicrobials across two time periods. Particularly worrying was the high level of resistance in the commonly used ciprofloxacin against *S. aureus.* All the Gram-positive isolates tested were susceptible, however, to teicoplanin and the novel antimicrobials meropenem and linezolid. Lower resistance was seen against the Gram-negatives tested. Overall, this data suggest that of the antimicrobials currently in use, moxifloxacin offers the best coverage against both Gram-positive and -negative isolates. Of the newer antimicrobials potentially suitable for ophthalmic use, meropenem appears to be a good choice for empiric monotherapy in bacterial keratitis, offering broad-spectrum cover against both Gram-positive and -negative microorganisms, with only one of the 772 isolates tested (*P. aeruginosa*) found to be resistant. Linezolid was active against all *S. aureus* isolates including a small number of MRSA isolates. Linezolid may therefore be useful for dual therapy when used in

combination with an antimicrobial with good cover against Gram-negative bacteria.

Antimicrobial combinations were investigated for synergy or antagonism against isolates of *S. aureus* and *P. aeruginosa*. Isolates were collected from cases of microbial keratitis from six centres in the UK. E-Test strips were used to test selected antimicrobials in combination against a representative set of 10 *S. aureus* and 10 *P. aeruginosa* isolates. Antimicrobial combinations were classified as synergistic, additive, indifferent, or antagonistic, according to their fractional inhibitory concentration (FIC). The combinations meropenem and ciprofloxacin, meropenem and teicoplanin, moxifloxacin and teicoplanin and ciprofloxacin and teicoplanin, gave the lowest mean FICs for *S. aureus*, with synergy or additivity being seen in 60% to 80% of isolates. The meropenem/ciprofloxacin combination gave the lowest mean FIC for *P. aeruginosa* isolates, with 90% showing an additive or synergistic effect. The other combinations elicited a predominantly indifferent response. No consistent antagonistic effect was observed with the combinations used. In conclusion, the combination of meropenem and ciprofloxacin was predominantly additive or synergistic for both *S. aureus* and *P. aeruginosa*. Teicoplanin combined with meropenem, ciprofloxacin, or moxifloxacin was also predominantly additive or synergistic against *S. aureus*.

Following the above two studies, the antimicrobial meropenem was selected for further corneal pharmacokinetic investigation.

6.32 Pharmacokinetics of meropenem

To investigate the toxicity of topically applied meropenem, corneal cell toxicity assays were undertaken using cultures of HKs and HCEs. MTT and Live Dead assays evaluated cell viability of cells treated with meropenem and moxifloxacin.

Corneal penetration of meropenem was assessed in human cadaver corneo-scleral discs mounted onto artificial anterior chambers. Meropenem 10 mg/ml was applied and samples collected at 45 mins, 1.5 hrs, 3.5 hrs and 24 hrs. Concentrations of meropenem were estimated using (1) a bioassay with *E. coli* and (2) HPLC. MTT assay showed meropenem had significantly higher cell viability at both 5 mg/ml and 2.5 mg/ml compared to moxifloxacin ($p<0.05$). Live dead assay showed no statistical difference in cell viability between cells treated with meropenem and untreated controls. The concentration of meropenem in the aqueous exceeded the MIC_{90} at 45 mins; 1.3 µg/ml (±1.5) bioassay and 0.9 µg/ml (±0.9) HPLC and steadily increased over 24 hours to; 43.7 µg/ml (±27.2) bioassay and 13.5 µg/ml (±4.8) HPLC. At the 24 hour time point, the meropenem concentration measured by bioassay exceeded measurements made by HPLC in 12 out of the 18 corneas. This may be due to metabolites of meropenem that are active in the bioassay but not quantifiable by HPLC.

In conclusion meropenem was shown to have low cellular toxicity on HKs and HCEs. It had good tissue penetration, achieving concentrations well above the MIC_{90} by 45 minutes.

6.4 Bacterial keratitis: the future

A small proportion of patients with keratitis continue to have a poor outcome when managed with conventional topical antimicrobial therapy. Difficulties in bacteriological diagnosis, increasing antimicrobial resistance, and poor pharmacokinetics are all drawbacks to standard techniques in managing the disease. Potential areas of further research will be now be discussed; firstly, novel strategies in diagnosing keratitis and secondly novel strategies in treating keratitis.

6.41 Novel strategies in diagnosis

In order to determine the causative bacteria in a patient with keratitis, samples from the corneal ulcer need to be collected. This is conventionally undertaken using a blade or a needle to scrape the edges of the ulcer which are then inoculated directly onto agar plates (figure 9). Kaye et al [61] reported that collecting two corneal scrapes, one for a smear and the other placed in BHI broth, resulted in detection rates similar to those of direct plating with no significant loss of organisms (chapter 1.131). Culture yield from corneal ulcers using corneal scraping, however, are low varying between 30 to 60%.[61] This may explain the reluctance of some ophthalmologists to perform a corneal scrape to reach a microbiological diagnosis. For example, McDonnell et al [330] found that 49% of ophthalmologists treated corneal ulcers empirically without attempting to identify the causative organism. It is evident that improvements are required in the detection and diagnosis of the causative bacteria in cases of suspected bacterial keratitis.

6.411 Impression cytology

The application of circular pieces of cellulose filter paper onto the ocular surface, known as impression cytology (IC), has been shown to reliably remove surface cells from the eye in viral,[331, 332] fungal,[333] and acanthamoeba[334] corneal infections. It has also been investigated in the diagnosis of a variety of other ocular surface conditions such as ocular surface neoplasia,[335] keratoconjunctivitis sicca,[336] vitamin A deficiency,[337] and atopic conjunctitivits.[338] This technique is simple to perform and less traumatic than the conventional scraping method. Furthermore it is potentially less invasive for the patient and is easier to apply

than using a blade to scrape the ulcer. IC may therefore, be suitable for use by non-ophthalmologists or where less sophisticated biomicroscopes are available, such as in less developed countries. In addition IC allows collection of fluid overlying the ulcer, which can then be analysed for bacterial secreted factors including QS molecules. Lastly, an additional but overlooked issue is the ability to determine or measure the bacterial response to treatment. By sampling the corneal ulcer during the time course of infection with IC it will be possible to study bacterial adaptations to treatment. This will provide a clear picture of bacterial mechanisms during *in vivo* infection and help to identify appropriate novel treatment strategies.

A randomised control study comparing the application of IC filter paper to the standard corneal scrape method is currently in progress. This will ascertain the value of IC in diagnosing bacterial keratitis.

6.412 Molecular biology techniques

Recently attempts have focused on the diagnostic yield from bacterial DNA using PCR to amplify bacterial DNA from patients with keratitis.[63, 339] This involves either amplifying a conserved region of DNA from the gene encoding the bacterial ribosomal RNA and then sequencing the product to identify the type of bacteria, or using a set of primers specific to known bacterial pathogens. Subrayan et al[339] evaluated the role of real-time PCR in the detection of *P. aeruginosa* in 10 patients with keratitis. They found PCR was at least as good as conventional cultures in detecting *P. aeruginosa*. Larger, randomised control trials, however, would be necessary to fully ascertain the role of PCR in diagnosing keratitis.

Matrix Assisted Laser Desorption/ Ionisation - Time Of Flight (MALDI-TOF) is also being used in increasing frequency to identify bacteria in other

infectious diseases.[340, 341] MALDI-TOF uses the application of a matrix to break open the bacterial cell wall which immediately crystallizes proteins. A laser generates a cloud of ions which are accelerated up a tube in the analyser. The detector estimates the molecular weight of the ions by calculating the time of flight it takes for the ions to reach the detector. A protein profile is thus produced, which can be compared to a database to identify the bacteria.

Utilising molecular techniques detecting DNA such as PCR and/or MALDI-TOF, may improve the diagnostic yield in patients with keratitis and also has the advantage of being a quicker and more sensitive technique than traditional culture methods. The interpretation of these DNA detecting techniques, however, may be difficult, because the presence of bacterial DNA does not distinguish between an active or inactive infection. To determine the degree of bacterial activity, and importantly the expression of virulence factors, the detection and measurement of bacterial mRNA would complement the use of DNA methods and this may be the future direction in diagnosing the disease.

Molecular biological methods also provides the opportunity to identify particular sub-sets of bacteria associated with keratitis (chapter 3 and 4). Early detection of virulence factors such as *lukSF*-PV, could provide information to enable treatment of bacterial keratitis to be modified if necessary. Knowledge of particular virulence factors may enable a clinician to predict if a patient is at higher risk of experiencing a poorer prognosis. This will help target such patients for more intensive treatment including hospital admission, frequent antimicrobial therapy and earlier surgical intervention.

6.42 Novel management strategies

As discussed in chapter 5.13, increasing resistance to commonly prescribed antimicrobials treating keratitis has been noted. In order to combat this, various strategies have been proposed; (1) to develop existing antimicrobials that have not yet been used in the eye, (2) to develop more effective ways of drug delivery and (3) to tailor drugs against specific virulence factors in selected patients.

6.42 Novel strategies in management

6.421 Development of existing antimicrobials

Sueke et al[104] investigated a number of antimicrobials that had previously been used to treat systemic infections, but had not been used (other than an occasional case report for some[342]) as a topical application to treat microbial keratitis or other ocular infections. Meropenem has been discussed in detail in chapter 5. Linezolid and tigecylcline were also suggested by Sueke et al[104] as potential novel antimicrobials for bacterial keratitis.

Linezolid,[343] the first of a new class the oxazolidinones, is a synthetic compound with activity against all the major Gram-positive groups of bacteria, but no activity against Gram-negative bacteria. Linezolid works by inhibiting bacterial ribosomal protein synthesis by binding to a site on the 50S ribosomal subunit thus preventing the formation of a 70S initiation complex. The investigation of MICs in chapter 5.41[104] showed linezolid to have low MICs against Gram-positive isolates including MRSA. Pharmacokinetic studies using animal models of keratitis have showed good corneal penetration and no recorded toxicity with linezolid.[344, 345]

Tigecycline[346] is a glycylcycline with activity against most aerobic and anaerobic Gram-positive and Gram-negative bacteria, but with limited activity

against *P. aeruginosa.* Glycylclines are bacteriostatic agents that inhibit protein synthesis in bacteria by reversibly binding to the 30S ribosomal subunit. Sueke *et. al.*[104] showed Gram-positive isolates to have no resistance to tigecycline using systemic breakpoints, but *P. aeruginosa* had 100% resistance. Corneal pharmacokinetics of tigecycline have not yet been determined.

6.423 Drug delivery systems

The majority of ophthalmic drugs used to treat keratitis are administered to the eye topically in the form of drops, solutions, emulsions or suspensions. It is well known, however, that this delivery method is inefficient and the majority of drug delivered topically never reach the aqueous humour. This may in part account for the poor outcome from bacterial keratitis.[251] Zhang et al[347] suggests that less than 5% of lipophilic molecules reach the aqueous humour and less than 0.5% of hydrophilic molecules. Reasons for this low efficiency include clearance by tear drainage and the presence of the corneal barrier. Patient compliance also affects the success of topical administration.

Many studies have investigated means of increasing drug absorption when the agent is delivered topically. This area has recently been reviewed by Shirasaki et al.[348] One method is to make the drug more lipophilic so it will pass more easily through the cornea. Increasing the solubility of the drug can also increase penetration. Methods of modifying the physicochemical properties of drugs to increase ocular penetration may include the addition of particulates such as nano-particles and other penetration enhancers, as well as using the prodrug and mucoadhesive dosage forms. Iontophoresis is a novel method improving ocular penetration of topically applied drugs.[349] It is a non-invasive technique where a

small electric current is applied to enhance ionized drug penetration into tissue. The drug is applied with an electrode carrying the same charge as the drug, and the ground electrode, which is of the opposite charge, is placed elsewhere to complete the circuit. The drug serves as a conductor of the current through the tissue. Eljarrat-Binstock and Domb[349] have reviewed the use of iontophoresis in ocular drug delivery.

Some researchers have looked at alternative drug delivery devices to enhance antimicrobial delivery (summarised in table 19).[251, 350] They broadly fall into two groups: matrix and reservoir based devices. In matrix-based implants, such as Bioadhesive Ophthalmic Drug Inserts,[351] the drug is distributed throughout a polymer matrix. The polymer is usually degradable, with common components being polylactic acid, polyglycolic acid and polylactic-co-glycolic acid. Once the matrix is introduced to the eye, the degradation of the polymer leads to the release of the drug, so tailoring of the degradation rate controls the rate of drug release.

In a reservoir implant, the drug is stored within a reservoir and is generally released over a time period of months or years. Non-degradable reservoir implants, such as Ocufit SR®,[251] are often manufactured from a combination of polyvinyl alcohol (PVA) and ethylene vinyl acetate (EVA). In a standard reservoir design, the drug is formed into a pellet with PVA and the pellet is coated with EVA. In a reservoir implant the drug is stored within a reservoir made of a non-degradable substance such as a collagen shield.

Table19. Summary of some of the drug delivery devices that have been trialled clinically. M: Matrix implant; NI: Nonimplantable; PLGA: Polylactic-co-glycolic acid; PVA: Polyvinyl alcohol; R: Reservoir-based implant.

Delivery system	**Insertion**	**Type and location**	**duration**
Soluble Ophthalmic Drug Insert (SODI™)	Upper/lower conjunctiva	NI, M film of acrylamide, *N*-vinylpyrrolidone, ethyl acrylate	Hours
Lacrisert®	Lower conjunctiva	NI, M Hydoxypropyl cellulose	1 day
Bioadhesive Ophthalmic Drug Inserts (BODI®)	Lower conjunctiva	NI, M Combination of 4 polymers	Days
Ocufit SR®	Lower conjunctiva	NI, R Silicone elastomer tube	weeks
Scleral plugs	Sclera	I, M biodegradable polymers, PLGA	1 month

6.424 Anti-virulence therapy

Traditional approaches to antimicrobial therapy are based on targeting bacterial cellular processes crucial for survival (chapter 5.12). Reducing bacterial survival, however, has two major drawbacks; (1) the encouragement of antimicrobial resistance (chapter 5.13) and (2) the non-intended eradication of useful host symbiotic bacteria.[352]

Targeting bacterial virulence is an alternative approach in treating infectious disease, which may offer a reduced selection pressure for drug-resistant mutations. This strategy aims at disarming pathogens of their harmful properties, without threatening their existence.[353] Anti-virulence drugs could be designed to

target specific pathogens and the virulence factors that are unique to their pathogenic cascades. As discussed in chapter 1.33, bacteria use an array of virulence factors to cause disease including adhesins, toxins, specialized secretion systems and QS. These are all potential therapeutic targets.

Many Gram-positive and Gram-negative bacteria use a pilus to adhere to the host cell (chapter 1.33). Pilicides are mimics of the normal pilin subunits that, when incorporated into the growing pilus, prevent elongation and the formation of a functional pilus. Treatment with pilicides has been shown to decrease the efficiency of colonization of *E. coli.*[354]

Inhibitors to bacterial toxins has been extensively investigated *in vitro* and *in vivo.* For example, an inhibitor of Las B (a metalloproteinase secreted by *P. aeruginosa* chapter 4.122) has been shown to inhibit targets that are instrumental in biofilm formation and immunomodulation *in vitro.*[355] McCormick et al[356] demonstrated that inhibition of α toxin associated with *S. aureus* (chapter 3.122) resulted in significantly less corneal damage in a rabbit model of keratitis.

The importance of the TTSS in the virulence of *P. aeruginosa* has been discussed in chapter 4.122. Numerous small-molecule inhibitors of this system have been identified using high-throughput screening (HTS) of small-molecule libraries.[357]

Several strategies have been developed to inhibit the QS system. For example; the marine macroalgae *Delisea pulchra* has been shown to inhibit the AHL-based QS system of *Pseudomonas.*[358] The inhibition of auto-inducing peptide that regulates the Agr QS in *S. aureus* (chapter 3.12) has also been suggested.[359] Although these molecules show promising *in vitro* results in preventing pathogenesis, *in vivo* activity remains to be assessed.

Safety is always a concern when introducing new therapies. As anti-virulence treatments are targeted to pathways or factors that exist exclusively in pathogens, it is likely that there will be less host toxicity compared to therapies that affect targets which are also expressed by the host. It is unclear, however, what effects these types of therapies will have on the commensal bacterial population and whether they will produce metabolites harmful to the host. Broad-scale clinical trials would shed light on this question.

6.5 Conclusion: patient stratification and personalised treatment

New approaches are necessary in managing patients with the sight-threatening disease bacterial keratitis.

This study has identified virulence factors and genetic characteristics have been identified in *S. aureus* and *P. aeruginosa* associated with keratitis. Future strategies in the disease may involve the identification of particular virulence factors that may enable the (1) the stratification of patients into poor and good outcomes and (2) the use of specific anti-virulence therapy in selected patients causing less side effects and resistance.

Using a pharmacodynamic / pharmacokinetic approach, this study has identified meropenem as an ideal candidate for the treatment of keratitis. The antimicrobial was shown to have; low MICs, good synergistic properties in combination with other antimicrobials, adequate corneal penetration and low cellular toxicity. Further studies on live human subjects would be necessary prior to initiating it as a therapy in disease.

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APPENDIX

(A) LB broth

Components

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

All components were dissolved in 1L of sterile distilled water and mixed to dissolve, sterilised by autoclaving at 121 °C for 15 mins, cooled and stored at room temperature.

(B) Molecular techniques

1) Array Tube Solutions and Buffers

Wash buffer 1 (2xSSC/0.01% Triton X100)

NaCl	175.3 g
Sodium citrate	88.2 g

Nacl and sodium citrate were dissolved in 800 ml of sterile distilled water (SDW). The pH was adjusted to 7.0 and the volume made up to 1L with SDW. The solution was then diluted 1:10 in H_20 and Triton X100 was added to a final concentration of 0.01% v/v.

Wash buffer 2 (0.2 x SSC)

Wash buffer 1 was diluted 1:100

Hybridisation buffer

Formamide 60-100% 125 ml

HRP Conjugation solution

Luminol (3-aminopthalhydrazide) 125 ml

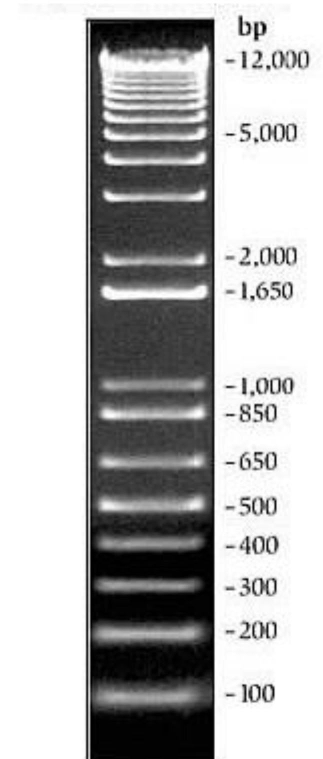
2) TBE Buffer

Tris	162 g
EDTA	11.16 g
Boric Acid	83.5 g

All components were added to 2.5 litres of distilled water. This was then mixed on a stirring plate until completely dissolved. The volume was then made up to 3 litres.

3) DNA ladders/Markers

1 kb plus DNA ladder (Invitrogen)



The 1 kb plus DNA ladder is composed of 20 double-stranded DNA bands ranging from 100 bp to 12,000 bp. This ladder has 12 evenly spaced bands ranging from 1 kb to 12 kb, a quick orientation band at 1,650 bp that forms a distinct doublet with the 2 kb band, and seven bands of round sizes below 1 kb. The ladder contains: 1 Kb Plus DNA Ladder™ (1 µg/µl) in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA. For loading 1 part 1 Kb plus ladder was added to 5 parts 6x loading buffer.

(C) MLST gene sequences

1) arcc

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATG

CCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATTGG

TTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGA

ACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGATCCA

CGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAA

GAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGA

AGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCA

ATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAA

TATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGA

AAATACCTATGAAGGTGTTGAAGCG

2) aroe

AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTC

CAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT

TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGT

ATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA

CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTA

TTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATG

CATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTT

ATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAGAA

CGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATT

AGCAGATGCTGAAAAGTATTTA

3) glpf

GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACA

ATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACCCAG

CGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGT

TCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAGCAACA

ATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCT

GGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAGAATTAC

TTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAG

GTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAAATCCTTT

AATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGC

TACTGGTTATGCAATCAACCCAGCACGT

4) gmk

CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATG

ACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTTT

TTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTT

ATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCAA

TATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATT

GAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCGCT

ATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTA

GTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAA

CGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA

5) pta

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGT

GATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATATT

TCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAA

TTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAGAA

CAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTT

GTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACATTCA

ACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCA

GGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGATGTA

CAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAA

GGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA

6) tpi

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAA

ACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAGCG

TGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAG

CTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGCTTA

TGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGA

TGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACTTATCA

AGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTT

AAACCTAACAACATTAAAGAATACATGGCACAAACTGATATTGATGG

GGCATTAGTAGGTGGCGCA

7) yqil

GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAA

CATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAGTT

ATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACG

AATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTAC

AGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATA

TCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGGTAT

GGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGG

TTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATGATGG

TTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAAT

TTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACATTTGCT

GTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA

(D) Clone types of *P. aeruginosa* isolates

Strain	Source	Hexa code[a]	Clone type[b]
96004	Bristol	4822	novel[c]
96009	Bristol	F469	D
96024	Bristol	F4A9	novel[c]
96034	Bristol	F429	I
96035	Bristol	F429	I
96036	Bristol	D421	A
96037	Bristol	F469	D
96043	Bristol	1BAA	novel[c]
96044	Bristol	2422	novel[c]
96045	Bristol	E429	B
96046	Bristol	FE69	novel[c]
96056	Bristol	D421	A
96062	Bristol	F469	D
96063	Bristol	2F82	novel[c]
96064	Bristol	3C2A	U
96066	Bristol	E429	B
96074	Liverpool	C40A	C
96076	Bristol	3C2A	U
106003	Bristol	D421	A
106011	Liverpool	2598	novel[c]
106012	Liverpool	C40A	C
106019	Liverpool	2C22	novel[c]
106022	Bristol	042E	novel[c]
106026	London	D421	A
106028	London	0C8A	novel[c]
106029	London	0812	V
106033	London	F421	A2
106042	London	B428	novel[c]
106044	London	D421	A
106053	London	F469	D
106054	London	EC2A	J

106055	London	2F82	novel[c]
106056	London	F460	novel[c]
106057	London	85AA	A4
106065	London	0812	V
106068	London	EA0A	A3
106074	Bristol	9421	novel[c]
106075	Bristol	EC29	novel[c]
106080	Manchester	E429	B
106083	Manchester	4612	novel[c]
106094	Bristol	F421	A2
106103	Birmingham	3C1A	novel[c]
106107	Bristol	C40A	C
106120	Bristol	D421	A
106122	Birmingham	FD9A	novel[c]
106123	Birmingham	241A	novel[c]
106124	Birmingham	C40A	C
106125	Birmingham	D421	A
106146	Bristol	C429	novel[c]
106147	Birmingham	ED9A	novel[c]
106149	Birmingham	E429	B
106151	Birmingham	C40A	C
106152	Birmingham	0812	V
106154	Birmingham	EC2A	J
106161	Bristol	EC21	novel[c]
106181	Bristol	2C22	novel[c]
106183	Birmingham	F429	I
106188	Liverpool	E429	B
106215	Bristol	4992	novel[c]
106221	Birmingham	2F02	novel[c]

a Hexadecimal code generated from AT SNP analysis
b Clone type identified in database
c Hexadecimal codes of novel clones were not assigned a clone type in database
na: not applicable

(E) Members of The MOG

Stephen Kaye, Craig Winstanley, Mal Horsburgh, Amanda Hall, Henri Sueke and Timothy Neal (Royal Liverpool University Hospital); Stephen Tuft (Moorfields' Eye Hospital); Derek Tole and John Leeming (Bristol Eye Hospital); Peter McDonnell (Birmingham and Midlands Eye Hospital); Francisco Figueiredo and Manjusha Narayanan (Royal Victoria Infirmary, Newcastle); Andrew Tullo, Fiona Carley, Hannah Lloyd and Malcolm Armstrong (Manchester Royal Eye Hospital); Colin Willoughby, Johnny Moore and Grace Ong (Royal Victoria Hospital, Belfast).