Current and Emerging Therapies for Corneal Infection: A Clinical and Laboratory Study

by

Dr. Darren Shu Jeng Ting

MBChB, PgCertHPE, DRCOphth, MRCOphth, FRCOphth

Medical Research Council / Fight for Sight PhD Fellow

This thesis is submitted to the University of Nottingham for the degree of Doctor of Philosophy (PhD)

May 2021

Imagination is more important than knowledge.

Knowledge is limited. Imagination encircles the world.

- Albert Einstein

ABSTRACT

Corneal infection or infectious keratitis (IK) is a major cause for corneal blindness worldwide. Broad-spectrum antimicrobial therapy is currently the mainstay of treatment for IK, but the efficacy is being challenged by the emergence of antimicrobial resistance. Host defense peptides (HDPs), also known as antimicrobial peptides (AMPs), are evolutionarily conserved molecules of innate immune system that are found in all kingdoms of life. HDPs have shown promise as a novel class of antimicrobial therapeutics due to their broad-spectrum and rapid antimicrobial activity against a wide array of infection with minimal risk of developing resistance. At the ocular surface, HDPs, particularly human cathelicidin (LL-37) and human beta-defensins (HBDs), have been shown to play a vital role during IK.

The first part of this work (Chapter 2 to Chapter 4) consisted of a body of work examining the epidemiology, causes, clinical characteristics, outcomes, and prognostic factors of IK in Nottingham, UK. IK was shown to be a persistent burden in Nottingham over the past decade, with ocular surface diseases, contact lens wear and systemic immunosuppression being the most common risk factors. More than 50% of the patients with IK required hospitalisation for intensive treatment, highlighting the burden of the disease on the patients and the healthcare system. Poor clinical outcome was significantly affected by older age, large infiltrate size and poor presenting vision.

The second part (Chapter 5 and Chapter 6) systematically examined the effectiveness and safety of adjuvant therapeutic corneal collagen cross-linking (PACK-CXL) and amniotic membrane transplant for treating IK, in addition to standard antimicrobial therapy. The meta-analyses demonstrated that both interventions significantly expedited the healing of IK, though the overall quality of

evidence was low, highlighting the need for further high-quality randomised controlled trials.

The third part (Chapter 7 and Chapter 8) highlighted a body of work in developing a new class of HDP-based antimicrobial therapy for IK based on hybrid derivatives of human cathelicidin (LL-37) and human beta-defensins-1 to -3. CaD23, derived from LL-37 and HBD-2, exhibited good *in vitro* efficacy against Gram-positive bacteria and moderate efficacy against Gram-negative bacteria. It demonstrated a rapid antimicrobial activity, which was likely attributed to its membrane-permeabilising activity, supported by SYTOX green dye uptake assay and molecular dynamics simulation study. CaD23 was also shown to exhibit a strong additive effect when used in combination with conventional antibiotics against Gram-positive bacteria. Finally, CaD23 exhibited good antimicrobial efficacy against Gram-positive bacteria (1.2 logCFU or 94% reduction in the bioburden) in a murine bacterial keratitis model. The discovery of CaD23 has provided a new scaffold for future development of newer generations of hybrid peptides.

ACKNOWLEDGEMENT

Ever since the early years of my medical career, I have always aspired to be a clinician scientist who excels in both clinical and research aspects. In 2018, I was at the verge of completing my 7-year ophthalmology specialist training where I had to decide whether to undertake an advanced surgical fellowship (as most trainees would do) or to take a 3-year break from my clinical training to undergo a formal PhD training. I am glad that I have chosen the latter, a bold decision which I would never regret making.

Coming from a purely clinically trained background, this 3-year PhD training programme serves as an eye-opening and much needed experience for me to become a clinical academic in ophthalmology. However, securing the initial PhD funding was certainly not a walk in the park. I am extremely thankful that I was with the right people in the right place at the right time. In 2018, I was very fortunate to be awarded the inaugural Fight for Sight / Royal College of Ophthalmologists (UK), John Lee Primer Fellowship, a scheme that aims to support early-stage ophthalmologists to undertake ground-breaking vision research. This Fellowship has also laid the foundation for my subsequent success in the Medical Research Council / Fight for Sight Clinical Research Training Fellowship. Nonetheless, all these Fellowships would not have been attained if I had not met my current supervisors, Professor Harminder Dua CBE and Dr. Imran Mohammed at Nottingham, who have played an instrumental role in guiding me through my Fellowship applications. Besides being my supervisor, I am grateful to have Prof. Dua, an inspirational world-leading figure, as my mentor, who is always ready to share his wisdom and knowledge with me in the clinical and research worlds, and beyond. I am also very thankful to Imran, who has provided me with all the bench side training and has transformed me from a green hand in the laboratory (i.e., never used a pipette before) to someone who can now

design and conduct own laboratory experiments. I also wish to thank Mrs Dalia Said, who is my clinical co-supervisor, for teaching and training me on both clinical and surgical aspects, and for helping me with many clinical research projects.

In addition, there are many more significant individuals whom I truly grateful and indebted to as the completion of this work would not have been possible without the help of them. I would like to thank my collaborators at Singapore Eye Research Institute (SERI), Professor Roger Beuerman and Associate Professor Rajamani Lakshminarayanan, who have been the key enablers of my MRC / Fight for Sight Fellowship application and have provided me with the essential training on the in vivo work related to corneal wound healing and bacterial keratitis studies. I am also grateful to all the team members in the anti-infective group of SERI, including Eunice Goh, Venkatesh Mayandi, Joanna Busoy, Barathi Veluchamy, Thet Aung, and Mercy Periayah, who have helped me with the microbiological studies, biophysical assays, and in vivo studies. I also wish to thank Dr. Jianguo Li and Prof. Chandra Verma at the A*STAR Bioinformatics Institute in Singapore for training and helping me with the molecular dynamics simulations studies. Whilst I was in Singapore, I have also been very fortunate to be guided by Professor Jodhbir Mehta, A/Prof Marcus Ang, A/Prof Daniel Ting (who also happens to be my elder brother), Gary Peh, and Yu-Chi Liu, who have kindly provided me with a lot of other research opportunities, allowing me to maximise my research experience beyond my PhD programme. This Nottingham-SERI collaboration is a fruitful relationship that I wish to continue for the rest of my clinical academic career.

I am also very grateful to Professor Ian Hall, who is the Director of the Nottingham Biomedical Research Centre and my mentor outside the world of ophthalmology, for providing me with a lot of insightful advice on my career path of becoming a clinical academic. I would also like to thank many of my colleagues and friends in Nottingham,

including Mouhamed Al-Aqaba, Ahmad Elsahn, Rashmi Deshmukh, Bina Kulkarni, Lydia Beekan, Rukhsar Akhtar, and Kostas Rallis, for helping me and making my work life in Nottingham enjoyable.

Finally, I would like to thank my family and my wife, Jiani, for being my pillar of strengths over all these years and for always believing in me.

Table of Contents

LIST OF ABBREVIATIONS

AD	Atopic dermatitis
AI	Artificial intelligence
AK	*Acanthamoeba* keratitis

AKC	Atopic keratoconjunctivitis
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
AMT	Amniotic membrane transplant
ANN	Artificial neural network
ANOVA	Analysis of variance
ARMOR	Antibiotic Resistance Among Ocular Microorganisms
ATCC	American Type Culture Collection
BK	Bacterial keratitis
CCK8	Cell-counting-kit 8
CDVA	Corrected-distance-visual-acuity
CECs	Corneal epithelial cells
CENTRAL	Cochrane Central Register of Controlled Trials
CF	Counting fingers
CFU	Colony forming unit
CI	Confidence interval
CoNS	Coagulase-negative staphylococci
CL	Contact lens
CLARE	Contact lens-induced acute red eye
CLPU	Contact lens-induced peripheral ulcer
CLSI	Clinical and Laboratory Standard Institute
CN	Culture-negative
CP	Culture-positive
CRAMP	Cathelin-related antimicrobial peptide
CXL	Corneal cross-linking
DAB	2,4-diamino-butyric acid
DAP	2,3-diamino-propionic acid

DED	Dry eye disease
DFT	Database-filtering technology
DPBS	Dulbecco's phosphate-buffered saline
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EPL	Ɛ-poly-L-lysine
ERK	Extracellular-signal-regulated kinase
HBD	Human beta-defensin
HDP	Host defense peptide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HM	Hand movement
HNP	Human neutrophil peptide
HSK	Herpes simplex keratitis
HZK	Herpes zoster keratitis
IACUC	Institutional Animal Care and Use Committee
ICTRP	International Clinical Trials Registry Platform
IIK	Interface infectious keratitis
IK	Infectious keratitis
IL	Interleukin
IQR	Interquartile range
ITT	Intention-to-treat
IVCM	In vivo confocal microscopy
KSFM	Keratinocyte serum free medium
LDH	Lactate dehydrogenase
LEAP	Liver-expressed antimicrobial peptide

LL-37	Human cathelicidin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen activated protein kinases
MD	Molecular dynamics
MDR	Multidrug resistant
MGD	Meibomian gland disease
MHA	Muller-Hinton agar
MHB	Muller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MUTT	Mycotic Ulcer Treatment Trial
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NC	Negative control
NGC	Negative Gaussian curvature
NGS	Next generation sequencing
NHS	National Health Service
NLR	Nucleotide-binding oligomerisation domain-like receptor
NPL	Non-perception of light
NRCS	Non-randomised controlled study
NTD	Neglected tropical disease
NTM	Non-tuberculous *Mycobacteria*
OD	Optical density
OS	Ocular surface
PACK-CXL	Photoactivated chromophore for infectious keratitis-corneal collagen cross-linking

PBS	Phosphate-buffered saline
PC	Positive control
PCR	Polymerase chain reaction
PL	Perception of light
PLGA	Poly lactic-co-glycolic acid
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analysis
QMC	Queen's Medical Centre
QoL	Quality of life
QSAR	Quantitative structure-activity relationship
RAGE	Receptor for advanced glycation endproducts
RCT	Randomised controlled trial
RNA	Ribonucleic acid
RNase	Ribonuclease
RR	Risk ratio
RSV	Respiratory syncytial virus
SAAP	Synthetic antimicrobial and antibiofilm peptides
SAR	Structure-activity relationship
SAT	Standard antimicrobial treatment
SCUT	Steroids for Corneal Ulcers Trial
SD	Standard deviation
SVM	Support vector machine
Tic-Oic	Tetrahydroisoquionolinecarboxylic acid - octahydroindolecarboxylic acid
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSA	Tryptone soya agar

UDVA	Uncorrected-distance-visual-acuity
UVA	Ultraviolet A
VKC	Vernal keratoconjunctivitis
WHO	World Health Organisation

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1. Background

The ocular surface (OS) is a specialised anatomical and functional system composed of various structures and components, including the tear film, conjunctival and corneal epithelium, lacrimal glands, meibomian glands, and nasolacrimal drainage apparatus (Gipson, 2007). Originating embryologically from the surface ectoderm, all these OS structures are linked anatomically via the epithelium and functionally via the regulation of neuronal, vascular, endocrinological, and immunological systems (Gipson, 2007). Together, they maintain the homeostasis of the OS which has critical roles in the optical quality of the eye to focus light at the retina as well as the most front-line defense system of the eye against a wide array of pathogens as well as physical and chemical insults (Ueta and Kinoshita, 2010). Any damage or insult to the OS, particularly the cornea, can result in pain and potentially permanent visual impairment or blindness.

Corneal opacity represents the 5th leading cause of blindness globally, accounting for approximately 3.2% of all cases (Flaxman et al., 2017, Ting et al., 2021j). The recent World Health Organisation (WHO) report highlighted that approximately 6 million of the world population are affected by cornea-related blindness or moderate/severe visual impairment, including 2 million of those who are affected by trachoma (Flaxman et al., 2017, Ting et al., 2021j). In addition, corneal opacity is estimated to be responsible for 1.5-2.0 million cases of unilateral blindness annually (Whitcher and

Srinivasan, 1997, Whitcher et al., 2001). These staggering figures underline the persistent and uncurbed burden of corneal blindness in the world, highlighting the need for innovative and drastic measures to tackle this issue.

1.2. Corneal infection

Any significant insult to the cornea such as infection, trauma, inflammation, degeneration, or nutritional deficiency can result in corneal opacity with visual impairment. Among all, corneal infection, also known as infectious keratitis (IK), has been shown to be the most common cause for corneal blindness in both developed and developing countries (Ung et al., 2019b, Ting et al., 2021j). According to a nationwide study, IK was shown to be the most common cause of all corneal blindness in China, primarily attributed to increased risk of trauma, low socioeconomic status, and illiteracy (Song et al., 2014). IK is a common yet potentially vision-threatening ophthalmic condition, characterised by acute ocular pain, decreased vision, corneal ulceration, and/or stromal infiltrates (Ung et al., 2019b). Previously, it has been recognised as a "silent epidemic" in the developing world (Whitcher and Srinivasan, 1997), and recently, a consortium-led proposal has suggested the designation of IK as a "neglected tropical disease (NTD)" (Ung et al., 2019a), adding on to the list of NTDs in ophthalmology (i.e. trachoma, onchocerciasis, and leprosy). The proposal to attain status of an NTD aims to draw concerted global effort to tackle IK in under-resourced tropical countries, to ameliorate the societal and humanistic burden of IK.

IK can be caused by a wide variety of pathogens including bacteria, fungi, protozoa, and viruses (**Figure 1.1**). In addition, polymicrobial infection has shown to be accountable for approximately 2-15% of all IK cases (Tan et al., 2017, Ting et al., 2018, Ting et al., 2021h, Khoo et al., 2020). As the OS is equipped with highly

regulated innate and adaptive defense mechanisms (Foulsham et al., 2018), IK rarely occurs in the absence of predisposing factors such as contact lens (CL) wear, trauma, OS diseases, and post-corneal surgery, which are some of the common risk factors implicated in IK (Khor et al., 2018, Ting et al., 2021j).

IK not only causes visual impairment, but also negatively impacts on the quality of life (QOL) of the affected individuals (**Figure 1.2**). A study from Uganda reported that IK affected both vision- and health-related QOL (Arunga et al., 2019). The psychological impact on these patients was related to the fear of losing the eye and the social stigma attached. Even when the visual recovery was complete, the individuals affected by IK displayed a lower QOL score than the unaffected controls (Arunga et al., 2019). Apart from the impact on the individuals which can affect their economic productivity, IK is also responsible for a huge economic burden on society. According to a report in 2010, the US spent an estimated 175 million dollars on the treatment of IK (Collier et al., 2014). Furthermore, complications of IK such as corneal perforations and scarring form the major indications of corneal transplants in developing countries such as India, Thailand and China (Khor et al., 2018), placing additional burden on the limited pool of donor corneas.

Considering that most parts of the world affected by IK are under-resourced, it is highly likely that the actual burden of IK is underestimated due to the lack of surveillance and under-reporting. This section aims to provide an updated and comprehensive overview of the epidemiology, causative microorganisms, risk factors, and the impact of antimicrobial resistance in relation to IK.

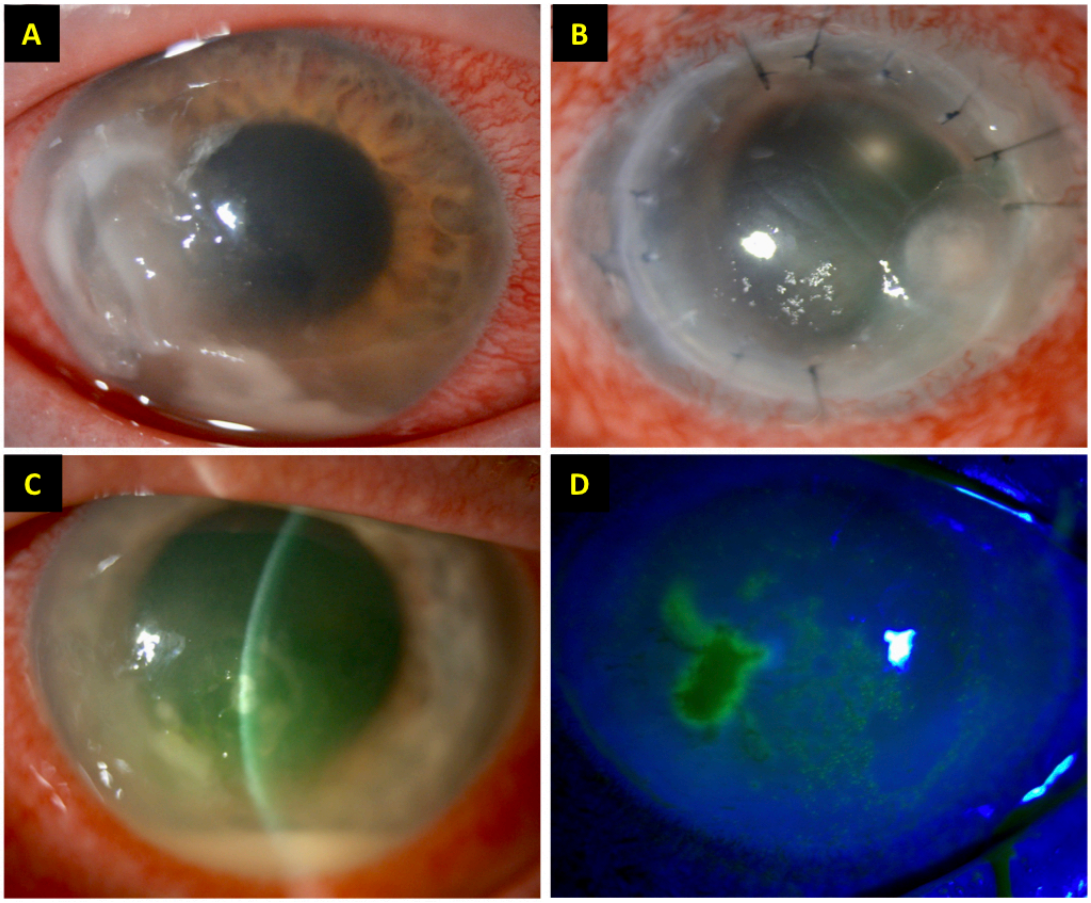


Figure 1.1. Different types of infectious keratitis.

Slit-lamp photographs demonstrating different types of infectious keratitis (IK).

(A) A case of IK caused by *Pseudomonas aeruginosa* in a contact lens wearer.

(B) A case of IK caused by *Staphylococcus aureus* due to a broken corneal graft suture.

(C-D) A case of polymicrobial IK, caused by *S. aureus* and herpes simplex keratitis, in a patient with atopic keratoconjunctivitis.

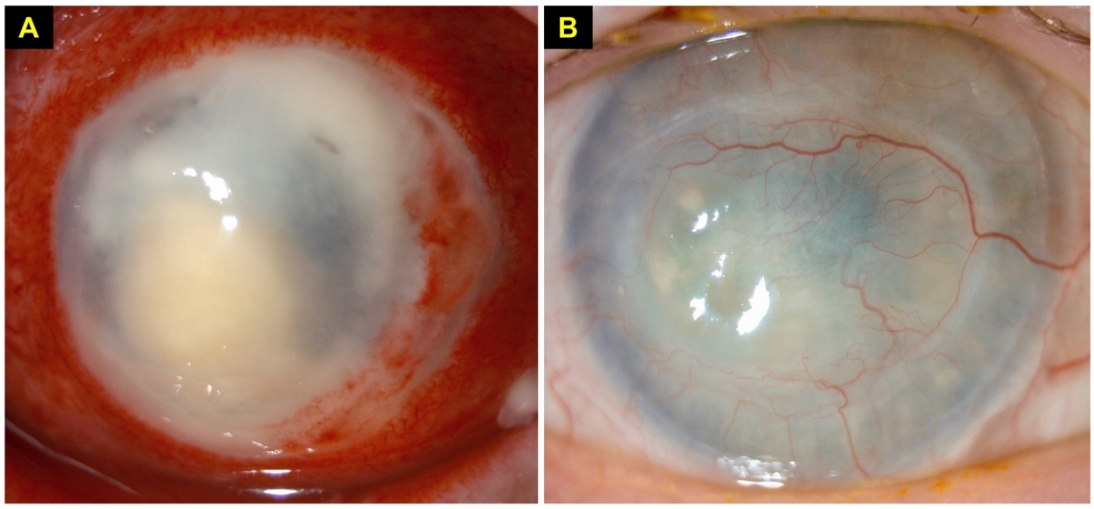


Figure 1.2. Clinical outcomes of severe infectious keratitis.

(A) Severe Pseudomonas aeruginosa-related bacterial keratitis complicated by scleritis and panophthalmitis, which required enucleation to eradicate the infection.

(B) Severe Acanthamoeba keratitis resulted in significant corneal scarring and intraocular inflammation, which required corneal transplantation.

1.2.1. Epidemiology

1.2.1.1. Incidence

To date, there are limited studies available in the literature that examined the incidence of IK and the majority of studies were conducted more than a decade ago (Ung et al., 2019b, Ting et al., 2021j). Depending on the geographical location and study design, the incidence of IK has been estimated to be in the range of 2.5-799 cases per 100,000 population/year (Erie et al., 1993, Upadhyay et al., 2001), particularly more prevalent in the low-income countries. Previous IK studies reported an estimated incidence of 2.5-27.6 per 100,000 population-year in the US (Erie et al., 1993, Jeng et al., 2010) and 2.6-40.3 per 100,000 population-year in the UK (Seal et al., 1999, Ibrahim et al., 2012). The recent Nottingham Infectious Keratitis Study concurred with the findings of these older studies. It was shown that the incidence remain relatively stable at a rate of 34.7 per 100,000 population-year in Nottingham, UK, between 2007 and 2019 (Ting et al., 2021h), highlighting a persistent burden of IK in the developed countries. Another recent study conducted in Australia similarly demonstrated a low IK incidence of 6.6 per 100,000 population-year during the period of 2005-2015 (Green et al., 2019a). However, it is noteworthy that the incidence reported in these two studies is likely to be underestimated as the numbers were based on IK patients who underwent corneal scraping.

In contrast, a substantially higher rate of IK has been reported in under-resourced countries such as South India (113 per 100,000 population-year) (Gonzales et al., 1996) and Nepal (799 per 100,000 population-year) (Upadhyay et al., 2001). The higher incidence observed in these regions was primarily attributable to the poorer environmental and personal hygiene, lower level of education, agricultural industry, increased risk to work-related corneal trauma, and poorer access to sanitation and healthcare facility.

1.2.1.2. Age

The epidemiological patterns and risk factors have been found to vary with demographic factors such as age, gender, and socioeconomic status. A tabulated summary of the demographic factors and microbiological profiles of IK is provided in **Table 1.1** (Kaye et al., 2013, Tan et al., 2017, Ting et al., 2018, Tavassoli et al., 2019, Ting et al., 2021h, Tam et al., 2017, Peng et al., 2018, Kowalski et al., 2020, Asbell et al., 2020, Cariello et al., 2011, Marujo et al., 2013, Hernandez-Camarena et al., 2015, Yu et al., 2016, Rautaraya et al., 2011, Lin et al., 2012, Kaliamurthy et al., 2013, Lalitha et al., 2015, Wang et al., 2015, Hsiao et al., 2016, Zhang et al., 2017b, Khor et al., 2018, Acharya et al., 2019, Lin et al., 2019, Politis et al., 2016, Cabrera-Aguas et al., 2019, Green et al., 2019a).

IK has been shown to affect individuals across all age groups. Based on large-scale studies (>500 patients), IK most commonly affected people aged between 30 and 55 years (**Table 1.1**) (Tan et al., 2017, Ting et al., 2018, Tavassoli et al., 2019, Ting et al., 2021h, Tam et al., 2017, Cariello et al., 2011, Hernandez-Camarena et al., 2015, Kaliamurthy et al., 2013, Wang et al., 2015, Politis et al., 2016, Zhang et al., 2017b, Khor et al., 2018, Cabrera-Aguas et al., 2019, Green et al., 2019a), primarily attributed to the underlying risk factors such as CL wear and ocular trauma associated with the working age group. Patients affected by trauma-related IK secondary to agricultural products and foreign bodies are usually around 45 to 55 years old (Keay et al., 2011, Jeng et al., 2010). The employed workforce of some developing countries is mainly composed of farmers and manual labourers, rendering them more susceptible to IK of traumatic aetiology (Khor et al., 2018, Chidambaram et al., 2018b). On the other hand, patients affected by CL-related IK are usually between 25 and 40 years old (Keay et al., 2011, Jeng et al., 2010, Tong et al., 2019, Stapleton, 2020).

Although prevalence of IK is generally low in the extremes of age (Ferreira et al., 2018b, Jeng et al., 2010, Ganguly et al., 2011, Al-Ghafri and Al-Raisi, 2018, Mandour et al., 2016), IK may serve as a major contributor to childhood blindness in some countries. For instance, IK was shown to be the second most common cause of visual impairment in children aged less than 15 years in Uganda (Waddell, 1998). Ophthalmia neonatorum, defined as conjunctivitis occurring in newborns within 28 days of life, is another important cause of childhood corneal blindness in developing countries, particularly when it is affected by *Neisseria gonorrhoea* where bilateral ocular involvement is common (Whitcher et al., 2001).

In addition, some studies have demonstrated that elderly patients affected by IK were associated with poor visual outcome (around 40-75% with visual acuity of <6/60) and higher rate of complications such as corneal melting, perforation and loss of eye (i.e. evisceration or enucleation) (Butler et al., 2005, Kunimoto et al., 2000, Khoo et al., 2020). This might be related to the higher rate of ocular co-morbidities and the delay in presentation and/or diagnosis of IK as elderly patients are usually dependent on spouse or family when seeking medical care and they may relate their condition to "normal" age-related changes (Barua et al., 2017, Srivastava and Gill, 2020).

1.2.1.3. Gender

The majority of studies did not observe any gender predilection in IK (**Table 1.1**). However, when gender difference or predominance exists, it is usually attributed to the underlying risk factors in different regions. For instance, CL-related IK has been shown to exhibit a female predominance of 57-69% (Keay et al., 2011, Tong et al., 2019, Jeng et al., 2010, Green et al., 2019b), whereas trauma-related IK is associated with a male predominance of 74-78% (Keay et al., 2011, Tong et al., 2019, Jeng et al., 2010), correlating with a high male prevalence (58-75%) of IK in the under-resourced regions such as South America (Cariello et al., 2011, Yu et al., 2016), Asia

(Ganguly et al., 2011, Pan et al., 2016, Chidambaram et al., 2018b, Khor et al., 2018), and Africa (Oladigbolu et al., 2013, Mandour et al., 2016, Zbiba and Abdesslem, 2018). Interestingly, a study in Nepal (Ganguly et al., 2011) found that there are significantly more male than female patients across all the age groups. This might be due to a combination of higher rate of trauma, lower number of CL wear, and reduced opportunities among the females to access medical services due to cultural customs.

1.2.1.4. Socioeconomic status and level of education

Low socioeconomic status has been shown to increase the risk of developing IK, primarily attributed to poor education, lack of ocular protection and personal hygiene, and limited access to eye care in rural communities (Chidambaram et al., 2018b, Khor et al., 2018, Mandour et al., 2016, Gautam et al., 2018, Song et al., 2014). In Asia and Africa, amongst those who were diagnosed with IK, approximately 45-71% of the patients were illiterate and 62-79% of them resided in rural areas with a poorer access to healthcare facilities (Kumar et al., 2011, Mandour et al., 2016, Zbiba and Abdesslem, 2018). In addition, it was found that farmers, rural residents and illiterates were at a higher risk of refractory IK with poorer outcomes (Mandour et al., 2016).

In some countries such as Nigeria and Malawi, residents in rural communities were shown to be more likely to self-medicate or approach village healers for traditional eye medicine (Oladigbolu et al., 2013, Courtright et al., 1994). Although it would be unfair to conclude that all therapies performed by traditional healers are inimical, common beliefs or practises of applying breast milk or plant products directly to the eye may actually worsen their keratitis (Courtright et al., 1994). In addition, patients who had prior use of traditional eye medicine tended to present later to the eye care professionals, resulting in delayed treatment and poorer visual outcome (Courtright et al., 1994).

Table 1.1. Summary of the demographic factors and microbiological profiles of infectious keratitis in the literature published between 2010 and 2020, categorised into six distinct regions. Only studies that reported more than 500 cases are included.

Year	Authors	Study period	Region	Total CS	Age (years)	Female (%)	Positive culture (%)	Organisms*			Microbiological profiles**
								B (%)	F (%)	A (%)	
UK and Europe											
2013	Kaye et al.	1995-2010	Liverpool, UK	2418	-	-	35.7	100	0	0	CoNS (26.3); *Enterobacteriaceae* (15.3); *Streptococci* (13.9)
2017	Tan et al.	2004-2015	Manchester, UK	4229	45.9	-	32.6	90.6	7.1	2.3	CoNS (24.4); *S. aureus* (15.1); *Streptococci* (13.3)
2018	Ting et al.	2008-2017	Sunderland, UK	914	55.9 ± 21.0	52.1	46.1	91.0	4.2	4.8	CoNS (25.9); *S. aureus* (13.6); Streptococci (12.1)
2019	Tavassoli et al.	2006-2017	Bristol and Bath, UK	2614	47.7 ± 21.2	51.1	38.1	91.6	6.9	1.4	CoNS (36.0); *Pseudomonas* (15.8); *Streptococci* (7.0)
2020	Ting et al.	2007-2019	Nottingham, UK	1333	49.9 ± 22.2	49.6	37.7	92.8	3.0	4.2	*Pseudomonas* (23.6); *S. aureus* (15.9); *Streptococci (*13.5)
North America											
2017	Tam et al.	2000-2015	Toronto, Canada	2330	41.6 ± 24.0	53	57.3	86.0	4.9	2.2	CoNS (37); *P aeruginosa* (10); *Streptococcus spp.* (15)
2018	Peng et al.	1996-2015	San Francisco, US	2203	-	-	23.7	100	0	0	*S. aureus* (20.1); *S. viridans* (13.2); *Pseudomonas* (10.9)
2019	Kowalski et al.	1993-2018	Pittsburgh, US	1387	-	-	100	72.1	6.7	5.2	*S. aureus* (20.3); *Pseudomonas* (18.0); *Streptococci.* (8.5)
2020	Asbell et al.	2009-2018	US	6091	-	46.8	100	100	0	0	*S. aureus* (35.9); CoNS (29); *H. influenza* (13)
South America											
2011	Cariello et al.	1975-2007	Brazil	6804	42.1 ± 21.4	40	48.6	78.9	11.0	3.6	CoNS (41.2); *S. aureus* (33.1); *Pseudomonas* (18.5)

2013	Marujo et al.	2005-2009	Brazil	2049	45	45	71.6	80.3	7.0	6	*Staphylococci* (52.5); *Corynebacterium* (14.3); *Streptococci* (10.1)
2015	Hernandez-Camarena et al.	2002-2011	Mexico	1638	45	51.4	38.0	88	12	0	*S. epidermis* (27.4); *Pseudomonas* (12.1); *S. aureus* (9.0)
2016	Yu et al.	1975-2010	Brazil	859	-	42.1	40.3	100	0	0	CoNS (23.8); *S. aureus* (20.9); *Pseudomonas* (14.2)
Asia											
2011	Rautaraya et al.	2006-2009	India	997	-	29.9	74.6	23.4	26.4	1.4	*Aspergillus spp.* (23.1); *Fusarium spp.* (19.2); *Staphylococci* (5.4)
2012	Lin et al.	2006-2009	India	5221	-	-	58	35.7	63.0	1.3	*Fusarium spp.* (15.5); *S. pneumoniae* (7.3); *Pseudomonas* (5.0)
2013	Kaliamurthy et al.	2005-2012	India	2170	45.7 ± 16.6	41.3	77	37.2	22.7	1.0	*S. epidermis* (44.0); *S. aureus* (19.5); *S. pneumonia* (11.6)
2015	Lalitha et al.	2002-2012	India	23897	-	-	59	24.7	34.3	2.2	*Fusarium spp.* (14.5); *Aspergillus spp.* (8.8); *S. pneumoniae* (7)
2015	Wang et al.	2013-2014	China	1000	-	31.8	53.5	0	100	0	*Aspergillus spp.* (53.8); *Fusarium spp.* (19.3)
2016	Hsiao et al.	2003-2012	Taiwan	2012	-	-	49.3	81.1	16	1.1	*Pseudomonas* (24.4); CoNS (16.6); *Propionibacterium* (9.1)
2017	Zhang et al.	2006-2015	China	6220	45.3 ± 22.1	40.6	18.2	100	0	0	*S. epidermis* (29.3); *P. aeruginosa* (11);
2018	Khor et al.	2012-2014	Asia	6626	46.0	39.2	70.7	38	32.7	-	*Fusarium spp.* (18.3); *Pseudomonas* (10.7); *Aspergillus flavus* (8.3)
2019	Acharya et al.	2015-2017	India	1169	-	-	100	100	0	0	CoNS (46.3); *Pseudomonas spp.* (16.2); *Streptococci* (15.5)
2019	Lin et al.	2010-2018	China	7229	-	-	42.8	52.7	57.6	0	CoNS (28.6); *Fusarium spp.* (23.5); *Aspergillus spp.* (12.2)

Africa and Middle East											
2016	Politis et al.	2002-2014	Israel	943	47.0 ± 25.2	47	47.9	91.8	8.2	0	CoNS (32.8); Pseudomonas (19.3); S. pneumonia (13.0)
Australasia											
2019	Cabrera-Aguas et al.	2012-2016	Sydney, Australia	1084	54	48	66	100	0	0	CoNS (45.8); *Pseudomonas spp*. (12.2); *S. aureus* (11.7)
2019	Green et al.	2005-2015	Queensland, Australia	3182	53 ± 22.6	47.6	73.6	93.1	6.3	0.6	CoNS (33.9); *Pseudomonas spp*. (17.7); *S. aureus* (11.2)

CS = Corneal scrapes; CoNS = Coagulase-negative staphylococci

*Breakdown of organisms; B = Bacteria, F = Fungi, A = Acanthamoeba

**The three most common microorganisms isolated in the study.

[$]Included all types of ocular infection.

[#]Included all types of ocular infection but restricted to bacterial infection only.

1.2.1.5. Seasonal variations

Pathogens are tremendously adaptive to climate and seasonality. Many studies have shown that IK was most prevalent during the summer season, with *P. aeruginosa* being one of the most frequently isolated microbes (Lin et al., 2012, Gorski et al., 2016, Walkden et al., 2018, Ting et al., 2021i). *P. aeruginosa* is a well-recognised organisms associated with environmental water as in swimming pools (Shankar et al., 2012) and CL solution (Dethorey et al., 2013, Ferreira et al., 2018b, Keay et al., 2011, Tong et al., 2019, Khor et al., 2020). The seasonal predilection of IK during summer is attributed to the likely increased use of CL wear and engagement in water activities. On the other hand, several studies have shown that the incidence of fungal keratitis in India peaked during the windy and harvest seasons, primarily related to a higher risk of trauma secondary to agricultural activities and agricultural debris being blown in the eyes by the wind (Lin et al., 2012, Kumar et al., 2011).

Seasonal variation was similarly observed in Acanthamoeba keratitis, though with conflicting results. Lin et al. (2012) observed that Acanthamoeba keratitis occurred more commonly during summer in South India, potentially related to the higher temperature and increased risk of corneal trauma during windy seasons, whereas Walkden et al. (2018) reported an increase in Acanthamoeba keratitis during the winter in the UK.

1.2.2. Causative microorganisms

A wide range of microorganisms, including bacteria, fungi, protozoa (particularly Acanthamoeba), and viruses, are capable of causing IK. A summary of large IK studies (>500 sample size) published during 2010-2020 is provided in **Table 1.1** (Kaye et al., 2013, Tan et al., 2017, Ting et al., 2018, Tavassoli et al., 2019, Ting et al., 2021h, Tam et al., 2017, Peng et al., 2018, Kowalski et al., 2020, Asbell et al.,

2020, Cariello et al., 2011, Marujo et al., 2013, Hernandez-Camarena et al., 2015, Yu et al., 2016, Rautaraya et al., 2011, Lin et al., 2012, Kaliamurthy et al., 2013, Lalitha et al., 2015, Wang et al., 2015, Hsiao et al., 2016, Politis et al., 2016, Zhang et al., 2017b, Khor et al., 2018, Acharya et al., 2019, Lin et al., 2019, Cabrera-Aguas et al., 2019, Green et al., 2019a).

1.2.2.1. Bacteria

Bacteria are commonly categorised into Gram-positive and Gram-negative bacteria based on the difference in the compositions of bacterial cell envelope. In addition to the universal structure of inner / cytoplasmic membrane, Gram-positive bacteria possess a thick outer cell wall, which is composed of layers of peptidoglycan interspersed with teichoic acids and lipotechoic acids, whereas Gram-negative bacteria consist of a thin middle-layer peptidoglycan and an additional outer membrane primarily made of lipopolysaccharide, which has been shown to play an important role in the pathogenesis of infection (including IK) and the contribution to host inflammatory responses (Silhavy et al., 2010, Fleiszig et al., 2020).

Bacterial keratitis represents the most common type of IK in most regions, including the UK (91-93%) (Tan et al., 2017, Ting et al., 2021h, Ting et al., 2018, Tavassoli et al., 2019), North America (86-92%) (Tam et al., 2017), South America (79-88%) (Cariello et al., 2011, Marujo et al., 2013, Hernandez-Camarena et al., 2015), Middle East (91.8%) (Politis et al., 2016), and Australasia (93-100%) (Cabrera-Aguas et al., 2019, Green et al., 2019a). In terms of specific bacterial strains, coagulase negative staphylococci (CoNS), which are a group of common ocular commensal (Becker et al., 2014), were shown to be the most commonly isolated organism (24-46%) in about half of the included studies (Kaye et al., 2013, Tan et al., 2017, Tam et al., 2017, Tavassoli et al., 2019, Ting et al., 2018, Cariello et al., 2011, Hernandez-Camarena et al., 2015, Yu et al., 2016, Kaliamurthy et al., 2013, Politis et al., 2016, Zhang et al.,

2017b, Acharya et al., 2019, Cabrera-Aguas et al., 2019, Green et al., 2019a, Lin et al., 2019). Other common bacteria implicated in IK included *S. aureus* (5-36%), *Streptococci* spp. (7-16%), *Pseudomonas aeruginosa* (5-24%), *Enterobacteriaceae spp.* (15%), *Corynebacterium spp.* (14%), and *Propionibacterium spp.* (9%; see **Table 1.1**). Interestingly, there were a few studies in the UK documenting a significant increase over the past decade, in the proportion of IK caused by *Moraxella spp.*, which are often associated with longer corneal healing time (Tan et al., 2017, Ting et al., 2021h, Ting et al., 2018).

Interestingly, *Nocardia* keratitis, a rare cause of IK, was identified as the third most common microorganism (11% of all cases) in the Steroids for Corneal Ulcers Trial (SCUT), and the outcome was found to be negatively influenced by the use of topical steroids (Lalitha et al., 2012b, Srinivasan et al., 2012). Acid-fast bacilli such as non-tuberculous mycobacteria (NTM) serve as another important group of pathogens that are capable of causing IK (Chu and Hu, 2013). NTM keratitis is commonly associated with refractive surgery and trauma, and it often requires prolonged and aggressive treatment for complete eradication, largely attributed to their propensity to form biofilms (Faria et al., 2015).

1.2.2.2. Fungi

Fungi can be broadly divided into two categories, namely filamentous and yeast or yeast-like fungi. Filamentous fungi such as *Fusarium spp.* and *Aspergillus spp.* normally thrives in tropical climates whereas yeast-like fungi such as Candida spp. were more commonly observed in temperate regions (Castano et al., 2020). Several studies have demonstrated that *Fusarium spp.* (13-24%) and *Aspergillus spp.* (8-30%) were the main causes of IK in Asia, particularly India and China (**Table 1.1**) (Lin et al., 2012, Rautaraya et al., 2011, Lalitha et al., 2015, Wang et al., 2015, Khor et al., 2018, Lin et al., 2019). In 2018, the Asian Cornea Society Infectious Keratitis

Study (ACSIKS) included more than 6000 patients from eight Asian countries and re-confirmed the dominance of *Fusarium spp.* keratitis within China (26%) and India (31%) established two decades ago (Leck et al., 2002, Sharma et al., 2002, Sun et al., 2004). Although the prevalence of fungal keratitis in temperate regions such as the UK, Europe and North America was reportedly lower, the growth of yeast-like fungi such as *Candida spp.* is relatively common in patients with history of corneal transplantation or OS diseases (Keay et al., 2011). In view of the recent improvement in the diagnostic techniques, rare pathogens such as *Cryptococcus curvatus*, *Arthrographis kalrae*, *Pythium spp.*, and many others are increasingly being identified and reported as rare causes of fungal keratitis (Ting et al., 2019c, Ting et al., 2020e, Sahay et al., 2020).

1.2.2.3. Protozoa

Acanthamoeba is a free-living protozoan that is found ubiquitously in the environment such as water, soil, air and dust (Somani et al., 2020). Although not as common as bacterial or fungal keratitis, Acanthamoeba keratitis (AK) serves as another important cause of IK as it is often associated with prolonged treatment course and poor visual outcome (Somani et al., 2020). It was estimated that AK affects 1-33 per million CL wearers per year (Somani et al., 2020). In the UK, Carnt et al. (2018a) recently confirmed an outbreak of Acanthamoeba keratitis in the South East England during 2010-2016, with an approximately threefold increase compared to the preceding decade.

Based on recent large studies, AK accounts for approximately 0-5% of all IK (**Table 1.1**). Most of the AK were observed in CL wearers (71-91%) (Dart et al., 2009, Yu et al., 2016, Zbiba and Abdesslem, 2018). However, non-CL wearers can also develop this infection if their eyes are exposed to contaminated water, soil or dust (Brown et al., 2018, Garg et al., 2017b). One of the Indian studies reported that only 4% of AK

cases were associated with CL wear and the remainder were associated with trauma and/or exposure to contaminated water (Garg et al., 2017b). In addition, the clinical features of non-CL related AK may differ from CL-related cases (Garg et al., 2017b). Moreover, Acanthamoeba sclerokeratitis may manifest as a rare but difficult-to-treat clinical entity that is usually associated with poor clinical outcomes (Iovieno et al., 2014).

Microsporidial keratitis represents another type of parasitic IK that accounts for approximately 0.4% cases of all IK (Moshirfar et al., 2020). It is mainly observed in Asian countries and may manifest as superficial keratoconjunctivitis or stromal keratitis. It is commonly associated with ocular trauma, exposure to contaminated water / soil, and potentially acquired immunodeficiency syndrome (Moshirfar et al., 2020, Friedberg et al., 1990).

1.2.2.4. Viruses

Viral keratitis, most commonly in the form of herpes simplex keratitis (HSK) and herpes zoster keratitis (HZK), represents a common cause of IK (Ting et al., 2019d, Ting et al., 2021j). However, as viral keratitis cases are commonly treated based on their typical clinical appearance (e.g. dendritic corneal ulcer in HSK) and/or previous ocular history, the majority of cases did not require any microbiological investigation and hence were not captured in many IK studies. Nonetheless, the ACSIKS study demonstrated that viral keratitis represented the most common cause (46%) of IK in China, primarily attributed to HSK (24%) and HZK (17%) (Khor et al., 2018). Another two studies, conducted in Egypt and China, respectively, observed that 15-21% of IK were caused by herpetic keratitis (Mandour et al., 2016, Pan et al., 2016). Based on these results, it is likely that viral keratitis represents an important and common cause of IK in many other regions, though further studies are required to elucidate this. Herpetic keratitis is often associated with neurotrophic keratopathy, which can result

in poor corneal healing, increased risk of further IK and other corneal complications such as melting and perforation (Ting et al., 2019d, Tuli et al., 2018).

1.2.2.5. Polymicrobial infection

Polymicrobial keratitis (IK caused by two or more causative microorganisms) has been reported in around 2-15% of all IK cases (Ting et al., 2021h, Ting et al., 2018, Tan et al., 2017, Green et al., 2019a, Khoo et al., 2020). Depending on the study design and the definition used, polymicrobial keratitis may include two or more types of organisms from the same category (e.g. bacteria-bacteria, fungus-fungus) or different categories (bacteria-fungus, fungus-Acanthamoeba). It is also noteworthy to mention that the incidence of polymicrobial keratitis is likely to be underestimated due to the variably low culture rate and the current conventional microbiological technique (i.e. microscopy and culture) may fail to unveil all the causative microorganisms in each IK case.

Polymicrobial keratitis often poses significant diagnostic and therapeutic challenges, and usually fares worse than monomicrobial keratitis (Ting et al., 2019c, Lim et al., 2013, Khoo et al., 2020). Khoo et al. (2020) observed that patients affected by polymicrobial keratitis (median of 6/60 vision) had a significantly worse visual outcome as compared to those affected by bacterial keratitis (median of 6/18 vision) or culture negative IK (median of 6/9 vision). In another retrospective comparative study, Lim et al. (2013) demonstrated that medical therapy was sufficient to resolve all monomicrobial IK cases but only 81% of polymicrobial IK. In view of the relatively common occurrence of polymicrobial keratitis and variably low culture yield of current microbiological investigation, clinicians should always maintain a low threshold of repeating corneal scraping if patients are not responding to either antibacterial or antifungal therapy, even in the presence of positive culture results.

1.2.3. Major risk factors

In the majority of IK cases, local and/or systemic risk factors are usually present. The most common risk factors include CL wear, ocular trauma, OS diseases (e.g. dry eye diseases, neurotrophic keratopathy, rosacea, etc.), lid diseases, post-corneal surgery (e.g. keratoplasty, corneal cross-linking), and systemic diseases (e.g. diabetes, immunosuppression), amongst others. A tabulated summary of large IK studies reporting the risk factors of IK is provided in **Table 1.2** (Dethorey et al., 2013, Ferreira et al., 2018b, Sagerfors et al., 2019, Jeng et al., 2010, Keay et al., 2011, French and Margo, 2013, Truong et al., 2015, Cariello et al., 2011, Yu et al., 2016, Kumar et al., 2011, Ganguly et al., 2011, Dhakhwa et al., 2012, Hussain et al., 2012, Deorukhkar et al., 2012, Kaliamurthy et al., 2013, Pan et al., 2016, Sitoula et al., 2015, Al-Ghafri and Al-Raisi, 2018, Chidambaram et al., 2018b, Khor et al., 2018, Gautam et al., 2018, Khor et al., 2020, Mandour et al., 2016, Oladigbolu et al., 2013, Tong et al., 2019, Khoo et al., 2020, Zbiba and Abdesslem, 2018).

1.2.3.1. Contact lens (CL) wear

CL wear has been recognised as one of the most common risk factors of IK, particularly in developed countries. A study conducted in Northern California reported that the incidence of IK among CL wearers was approximately 9.3 times higher than the non-CL wearers (130.4 vs. 14.0 per 100,000 person-years) (Jeng et al., 2010). Based on the large studies (>200 patients) published in the recent literature, CL wear was shown to be the main predisposing factor (29-64%) of IK in developed countries like Portugal (Ferreira et al., 2018b), France (Dethorey et al., 2013), Sweden (Sagerfors et al., 2019), the US (Jeng et al., 2010, Keay et al., 2011, Truong et al., 2015), Singapore (Tong et al., 2019), and Australia (Khoo et al., 2020). On the contrary, CL-related IK was considerably less common (0-18%) in developing countries due to less number of CL wearers (Oladigbolu et al., 2013, Khor et al., 2018,

Kaliamurthy et al., 2013, Al-Ghafri and Al-Raisi, 2018, Zbiba and Abdesslem, 2018), highlighting the geographical disparity in the risk factors as well as the causative microorganisms of IK between high income and low-income countries (**Table 1.2**).

The pathogenesis of CL-related IK is complex and multifactorial. Although it is commonly believed that CL-related IK is triggered by superficial injury secondary to CL wear, several studies have refuted this hypothesis as it is shown that the presence or absence of epithelial injury did not influence the risk or severity of IK (Fleiszig et al., 2020). Plausible mechanisms of CL-related IK include reduction of tear exchange during blinking (which leads to potential degradation of protective components at OS), tear stagnation under CL (particularly soft CL) resulting in accumulation and adherence of microbes to the cornea, reduced corneal epithelial cell desquamation, and alteration of tear fluid biochemistry (Fleiszig et al., 2020). In addition, multiple predisposing factors of CL-related IK have been identified, including the types of CL used (higher risk in soft CL than rigid gas permeable CL), poor CL and CL case hygiene, overnight wear, use of expired CL, types of CL solution used, and CL being prescribed / dispensed by non-ophthalmologists or non-opticians (Yildiz et al., 2012, Sauer et al., 2020, Carnt et al., 2018b, Stapleton et al., 2017b, Dethorey et al., 2013, Hoddenbach et al., 2014). Reports of IK secondary to the use of cosmetic lens and orthokeratology lens have also been highlighted (Sauer et al., 2016, Scanzera et al., 2020).

In terms of underlying aetiologies, CL-related keratitis is most commonly associated with *P. aeruginosa* and *Acanthamoeba spp*., which are both free-living microorganisms that are ubiquitously present in the environment, including water and CL solutions (Stapleton, 2020). As noted above, *Pseudomonas* keratitis is one of the most common causes of IK, especially in the developed countries where there is increased prevalence of CL wear. Yildiz et al. (2012) and Tong et al. (2019) observed

that *P. aeruginosa* was responsible for 63% and 70% of the CL-related IK, respectively. While AK is uncommon, most of these cases (71-91%) were observed in CL wearers (Dart et al., 2009, Zbiba and Abdesslem, 2018, Yu et al., 2016). Yu et al. (2016) observed that more than 90% of the AK were associated with CL use. In a 32-year Brazilian study of over 6000 IK cases, Cariello et al. (2011) reported that CL wearers had a 1.7 times higher risk of developing culture-positive AK than non-CL wearers. Interestingly, CL wear was also shown to be a major risk factor for fungal keratitis in a US study (Keay et al., 2011).

1.2.3.2. Trauma

Trauma serves as another common risk factor for IK in both developed and developing countries. Based on the IK studies reported in the literature, farmers (54-70%) and manual labour workers (11-17%) constituted the main occupations in Asia (Kumar et al., 2019, Ganguly et al., 2011, Pan et al., 2016, Chidambaram et al., 2018b, Mandour et al., 2016, Oladigbolu et al., 2013, Khor et al., 2018). These groups of workers were at a high risk of developing IK due to the increased occupational exposure to plant materials and foreign bodies, which was frequently compounded by the lack of eye protection (Chidambaram et al., 2018b, Kumar et al., 2019, Mandour et al., 2016, Pan et al., 2016, Dhakhwa et al., 2012).

Fungal keratitis was by far the most common cause (47-83%) of trauma-related IK, especially in regions such as Asia and Africa which are dominated by agricultural communities (Pan et al., 2016, Chidambaram et al., 2018b, Mandour et al., 2016, Zbiba and Abdesslem, 2018, Khor et al., 2020). Occupational exposures to vegetative matter, organic materials and animal products, predominantly in males in the working age group, are the main causes in these regions. The risk of fungal keratitis was further magnified by tropical climates, which are conducive to fungal growth (Mandour et al., 2016, Zbiba and Abdesslem, 2018). Cariello et al. (2011) observed that the risk

of developing culture-positive fungal keratitis was increased by 4 times if the patients suffered from plant-related trauma. In addition, some studies demonstrated that trauma-related IK fared worse than non-traumatic cases (Tong et al., 2019, Pan et al., 2016). Pan et al. (2016) conducted a 10-year study in China and revealed that patients who presented with trauma-related IK were at a high risk of developing fungal keratitis and requiring surgical interventions (89%), including therapeutic keratoplasty and evisceration / enucleation.

On the other hand, the majority of the trauma-related IK reported in European countries were caused by Gram-positive bacteria, including CoNS, *S.* aureus, *Streptococci,* and *Corynebacterium* (Ferreira et al., 2018b, Sagerfors et al., 2019). These are common OS commensals which have the ability to tolerate hot and dry climates in temperate and sub-tropical zones (Mandour et al., 2016, Suzuki et al., 2020, Graham et al., 2007). Corneal trauma resulting from non-vegetative matter with consequent secondary opportunistic infection with OS commensals could explain the high rate of Gram-positive infection in trauma-related IK in this region.

1.2.3.3. Ocular surface and eyelid diseases

OS diseases, encompassing dry eye disease (DED), blepharitis, neurotrophic keratopathy, Steven-Johnson syndrome, ocular cicatricial pemphigoid and bullous keratopathy, have been identified as one of the main risk factors for IK in both developed and developing countries (Khoo et al., 2019, Zbiba and Abdesslem, 2018, Truong et al., 2015, Ganguly et al., 2011, Keay et al., 2011, Jeng et al., 2010). OS disease-related IK is most commonly caused by Gram-positive bacteria (around 60-80%) (Sagerfors et al., 2019, Zbiba and Abdesslem, 2018, Khoo et al., 2020, Khoo et al., 2019), which constitute the main group of OS commensals. In particular, CoNS and *S. aureus* were shown to be the main culprits in OS disease-related IK (Khoo et al., 2019, Sagerfors et al., 2019, Ting et al., 2021a).

DED is the most common OS disease that is characterised by "*a loss of tear film homeostasis with ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles"* (Craig et al., 2017). The dysregulated OS health could lead to breakdown of the corneal epithelium, a vital OS defence, and OS inflammation, consequently increasing the risk of IK (Bron et al., 2017, Zbiba and Abdesslem, 2018).

Posterior blepharitis or meibomian gland disease (MGD) is a common eyelid disease which is difficult to cure. It can lead to an array of OS complications, including evaporative DED, marginal keratitis and IK, amongst others (McCulley and Shine, 2000). Meibomian gland abnormalities (e.g. gland dropout and hyperkeratinisation), alteration of the secreted lipid products, and the dysregulation of bacterial populations and their corresponding lipase or esterase activity are believed to contribute to the OS inflammation and infection. In a 5-year Australian study, MGD was shown to be the most common cause (79%) of OS disease implicated in IK (Khoo et al., 2019). In addition, nasolacrimal duct obstruction could also increase the risk of IK, primarily attributed to tear stagnation and reduction of tear exchange, resulting in the accumulation of microbes and debris on the OS with increased risk of IK. Chidambaram et al. (2018b) showed that nasolacrimal duct obstruction could increase the risk of fungal and bacterial IK, particularly *S. pneumonia* keratitis.

Table 1.2. Summary of risk factors and associated organisms of infectious keratitis in the literature published between 2010 and 2020, categorised into 6 distinct regions. Only studies that reported more than 200 cases are included.

Year	Authors	Study period	Region	Patients	Age, years (Mean ± SD)	Female, %	Risk factors (%)
UK and Europe							
2013	Dethorey et al.	2005-2011	France	268	45	50.4	CL (48.1), OSD (33.7), POS (17.5)
2018	Ferreira et al.	2007-2015	Portugal	235	50.0 ± 20.7	55.1	CL (28.9), trauma (28.9), DM (13)
2020	Sagerfors et al.	2004-2014	Sweden	398	49.5	57	CL (45.5), OSD (9.8), corneal transplant (9.5)
North America							
2010	Jeng et al.	1998-1999	US	302	42.8	57.3	CL (55), OSD (19.2), trauma (11.9)
2011	Keay et al.	2001-2007	US	733	47.9	46.8	CL (36.6), OSD (28.5), trauma (24.6)
2013	French et al.[#]	2010	US	2124	39.2	53.5	Scleral ectasia (4.8), CL (4.8), corneal abrasion (3.1)
2015	Truong et al.	2009-2014	US	318	42.9	40.3	CL (41), OSD (28), trauma (17), topical steroid (4)
South America							
2011	Cariello et al.	1975-2007	Brazil	16742	42.1 ± 21.4	40	POS (22.4), CL (12.8), trauma (16.4), topical steroid (6.6)
2016	Yu et al.	1975-2010	Brazil	859	-	42.1	Topical medication (30.6), Trauma (24), POS (24), CL (13)
Asia							
2011	Kumar et al.	2003-2005	India	200	-	39	Trauma (78.5), OSD (12)
2011	Ganguly et al.	2006-2007	Nepal	1880	-	40.7	Trauma (58), topical steroid (12), OSD (6), CL (5)
2012	Dhakhwa et al.	2007	Nepal	414	-	42.8	Farmers (75.4), trauma (33.3), topical steroid (4.1)
2012	Hussain et al.	2007-2009	Pakistan	228	42.8 ± 21.9	35.1	Trauma (31.5), POS (8.8), topical steroid (6.6)
2012	Deorukhkar et al.	2004-2009	India	852	-	31.7	Trauma (60.2), FB (15.6), POS (9.5)
2013	Kaliamurthy et al.	2005-2012	India	2170	45.7 ± 16.6	41.3	Trauma (64.0), traditional eye medicine (16.9)
2015	Sitoula et al.	2011	Nepal	1644	44 ± 16	42	Trauma (60), dacryocystitis (5)
2016	Pan et al.	2003-2012	China	578	52.4	25.4	Trauma (54.7), URTI (11.9), DM (8)

2018	Khor et al.	2012-2014	Asia	6563	46.0	39.2	Trauma (34.7), CL (10.7), POS (6.8), OSD (4.2)
2018	Chidambaram et al.	2012-2013	India	252	50	36	Trauma (71.8), traditional eye medicine (19.0) topical steroid (9.9), DM (6.7)
2018	Al-Ghafri et al.	2013-2016	Oman	304	52.2 ± 23.2	56.2	Blepharitis (54.3), trachoma (26.0), Other lid diseases (18.1), CL (17.1), Climate droplet keratopathy (15.5)
2018	Gautam et al.	2016	Nepal	259	44.9	54.4	Trauma vegetative material (48), topical steroid (9)
2019	Tong et al.	2012-2016	Singapore	377	33.6 ± 17.2	53.5	CL (64.3), OSD (10), trauma (3.9)
2020	Khor et al.	2010-2016	Malaysia	221	39.5	41.2	Trauma (49.3), CL (23.1), OSD (5.9)
Africa and Middle East							
2013	Oladigbolu et al.	1995-2005	Nigeria	228	-	43.4	Trauma (51.3), traditional eye medication (17.1), topical steroid (5.7)
2014	Mandour et al.	2010-2013	Egypt	340	-	41.2	Trauma (50), POS (14.7), topical steroid (11.8)
2018	Zbiba et al.	2011-2016	Tunisia	230	-	40	OSD (58.7), Trauma (51.3), DM (16), topical steroid (10.9), CL (9.5)
Australasia							
2020	Khoo et al.	2012-2016	Australia	979	54.7 ± 21.5	48.3	CL (63), topical steroid (24), OSD (18)

CL = Contact lens wear; POS = Previous ocular surgery; OSD = Ocular surface diseases; FB = Foreign bodies; DM = Diabetes; URTI = Upper respiratory tract infection

[#]The data was based on patients presented to general emergency department; therefore, risk factors might not be accurately documented.

1.2.3.4. Post-ocular surgery

IK may occur following various ocular surgeries, including corneal transplant, refractive surgery, corneal cross-linking (CXL), pterygium surgery, cataract surgery, and others (Cariello et al., 2011, Dohse et al., 2020, Maharana et al., 2018, Mandour et al., 2016, Song et al., 2021). Corneal transplant serves as the main sight-restoring surgery for a wide range of corneal diseases, though postoperative complications such as graft failure and IK may develop. In a retrospective study of over 2000 corneal transplants, Dohse et al. (2020) reported an incidence of post-keratoplasty IK of 4%, with loose and broken sutures being reported as one of the most common risk factors (24%). Cariello et al. (2011) demonstrated that 22% of the IK cases were associated with prior ocular surgery, particularly corneal graft (56%). In addition, the paradigm shift of penetrating keratoplasty to lamellar keratoplasty has created a new array of host-graft interface complications such as interface infectious keratitis (IIK), which often causes diagnostic and therapeutic challenges due to the deep-seated location of the infection (Ting et al., 2019g, Fontana et al., 2019). A clinically challenging case of post-endothelial keratoplasty interface fungal keratitis has also been recently highlighted, which required in vivo confocal microscopy for confirmatory diagnosis in the absence of positive culture results (**Figure 1.2**) (Ting et al., 2019g). Fortunately, the interface infection resolved quickly after the discontinuation of topical steroids and initiation of appropriate antifungal treatment.

Although IK rarely develops after refractive surgery, the significant amount of refractive surgeries performed globally render this an important clinical entity (Randleman and Shah, 2012). This was supported by a Brazilian study where refractive surgery was shown to be the second commonest surgery associated with IK (Cariello et al., 2011). Post-refractive surgery IK is most commonly caused by Gram-positive bacteria and non-tuberculous *Mycobacteria*, though fungal and Acanthamoeba infection may also occur (Randleman and Shah, 2012). The high rate

of Gram-positive bacterial IK following other types of ocular surgeries (e.g. cataract surgery, pterygium surgery) were also observed, most likely as a result of opportunistic infection secondary to OS commensals (Sagerfors et al., 2019, Dethorey et al., 2013, Mandour et al., 2016).

In the recent years, corneal cross-linking (CXL) has emerged as a therapeutic modality for managing corneal ectactic conditions (Elmassry et al., 2020, Ting et al., 2019f) and moderate-to-severe IK (Ting et al., 2019e, Prajna et al., 2020, Said et al., 2014). However, the intraoperative removal of corneal epithelium and postoperative insertion of bandage CL (which is the current standard practice in most institutes) can increase the risk of IK following CXL, particularly in patients with OS diseases such as vernal or atopic keratoconjunctivitis (Dhawan et al., 2011, Ting et al., 2019b, Maharana et al., 2018). Post-CXL IK may be further complicated by the reactivation of herpetic keratitis (Dhawan et al., 2011) and manifestation of acute hydrops (Ting et al., 2019b) and corneal melt/perforation (Maharana et al., 2018).

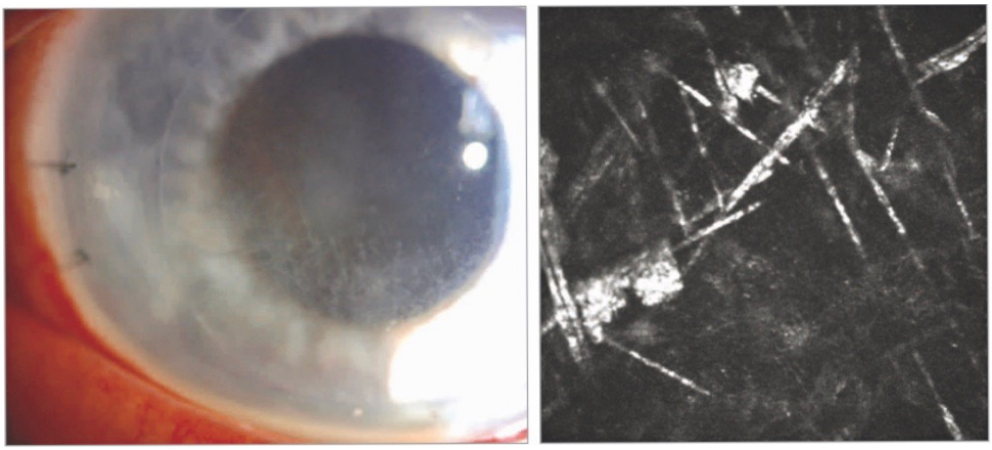


Figure 1.3. Interface infectious keratitis after DSAEK.

Slit-lamp photography demonstrating an inflamed right eye with diffused stromal haze in crisscross pattern at the graft-host interface and a mild opacity at the temporal host cornea. B, In vivo confocal micrograph demonstrating diffuse hyperreflective crisscross changes at the graft-host interface (at the depth of 598 μm). The image is in the scale of 400 × 400 μm. The figure is reproduced from a study published by Ting et al. (2019g).

1.2.3.5. Use of topical steroids

Steroids are commonly used in ophthalmology as a topical immunosuppressive / immunomodulatory agent to manage a wide range of intraocular and OS inflammatory diseases, including dry eye disease, allergic eye disease, non-infectious keratitis, chemical eye injury, cicatricial conjunctivitis, and many others (Jones et al., 2017, Tempest-Roe et al., 2013). The recent SCUT study also demonstrated the benefit of adjuvant topical steroids in improving the visual outcome in patients with severe and central bacterial keratitis (Srinivasan et al., 2012). In addition to managing OS diseases, topical steroids are also frequently used as postoperative topical treatment following intraocular and OS surgeries, including corneal transplantation (Di Zazzo et al., 2017).

However, topical steroids could sometimes act as a double-edge sword. Studies have shown that topical steroids could increase the risk of IK, particularly fungal keratitis and/or polymicrobial keratitis (Khoo et al., 2020, Keay et al., 2011, Ting et al., 2019g). In a study of 733 fungal keratitis, Keay et al. (2011) reported that 13% of the cases were associated with chronic use of topical steroids. In addition, a study has shown that previous use of topical steroids could negatively impact on the clinical outcome of IK, with 73% ending with poor outcome (defined as worse than 6/60 vision, decreased vision during treatment, or perforation) (Khoo et al., 2020). While topical steroids serve as an effective treatment for stromal HSK, which is primarily an immune-related keratitis (Wilhelmus et al., 2020), its use can potentially exacerbate epithelial HSK and culminate in geographic ulcer (Liesegang, 2001). Interestingly, an Indian study showed that 41% of the AK cases were associated with the use of topical steroids (Chidambaram et al., 2018b). The high rate of prior steroid use might be related to the fact that Acanthamoeba keratitis often presents with non-specific corneal epithelial changes and is mismanaged as viral keratitis (Carnt et al., 2018b).

1.2.3.6. Systemic immunosuppression

Systemic immunosuppression, either secondary to diseases or immunosuppressive agents, has been shown to increase the risk of IK. Diabetes mellitus serves as one of the most important systemic risk factors for IK. Hyperglycaemia has been shown to facilitate microbial growth and alter the microbiota of OS, including an upregulation of *Pseudomonas spp.* and *Acinetobacter spp*. (Li et al., 2019b), as well as affect the homeostasis, corneal sensation and wound healing of the corneal epithelium, thereby increasing the risk of IK (Zhu et al., 2019). Sub-basal corneal nerve plexus of patients with diabetic neuropathy is often affected and can lead to neuropathic keratopathy with complications such as corneal melt and IK (Al-Aqaba et al., 2019).

Several large studies have highlighted the association between diabetes and IK (around 8-16%), particularly fungal and bacterial keratitis (Dan et al., 2018, Pan et al., 2016, Chidambaram et al., 2018b, Zbiba and Abdesslem, 2018, Wang et al., 2018). Zbiba and Abdesslem (2018) observed that diabetes was relatively common in patients with bacterial keratitis (15%) and fungal keratitis (16%) as well as mixed bacterial and fungal keratitis (29%). In addition, viral keratitis was also reported to have a high prevalence amongst patients with diabetes (Kaiserman et al., 2005). Viruses, particularly HSV, are omnipresent in the general population, with an estimated prevalence of 1.5 per 1000 population (Liesegang, 1989). Kaiserman et al. (Kaiserman et al., 2005) demonstrated that patients with diabetes had a significantly higher incidence and recurrence rate of herpetic eye disease when compared to non-diabetic patients. Pan et al. (2016) observed that 17% patients with diabetes had a substantially higher rate of HSK as compared to bacterial or fungal keratitis. Another study described that all patients with diabetes presented with IK were of viral origin, though the sample size was small (Mandour et al., 2016). The heterogeneity in the subtypes of microorganisms associated with diabetes observed in different studies was likely related to the disparity in the ocular predisposing factors of the studied

cohort since more than one risk factor is often present in patients with IK (Khoo et al., 2020).

Apart from diabetes, Jeng et al. (2010) observed an approximately 10-fold increased risk of IK in individuals affected by human immunodeficiency virus (HIV) compared to healthy individuals (238.1 vs. 27.6 per 100,000 population-year), highlighting the importance of host immunity in OS defence. Intriguingly, a study demonstrated that 55% of the patients with HSK had a history of upper respiratory tract infection prior to the infection or recurrence (Pan et al., 2016). This could be potentially explained by the mechanism linked to a host cell enzyme called heparinase (Lobo et al., 2019), which is a known contributing factor to the pathogenesis of several viruses, including HSV, respiratory syncytial virus, human papilloma virus, and others. End-stage renal disease, particularly associated with diabetes, was also shown to be a risk factor for IK (Jan et al., 2018).

1.2.4. Antimicrobial resistance (AMR)

1.2.4.1. Overview

Antimicrobial resistance (AMR) has been recognised as a major public health crisis in the past two decades with many infectious organisms developing resistance against previously effective successful antimicrobial agents thus limiting therapeutic options (Laxminarayan et al., 2013). The development of AMR is largely driven by a multitude of factors, including the overuse / abuse of antimicrobial agents in agricultural sectors due to commercial pressure, uncertainty in diagnosis (e.g. bacterial infection vs. viral infection) leading to inappropriate use of antibiotics, financial incentives for prescribing antibiotic, and use of non-prescription antibiotics among the general public, particularly in low- and middle-income countries (Laxminarayan et al., 2013, Munita and Arias, 2016). From the genetic point of view,

bacteria primarily develop AMR through two strategies, namely genetic mutational resistance and horizontal gene transfer. The genetic and mechanistic basis of AMR can be referred to a recent excellent review provided by Munita and Arias (Munita and Arias, 2016).

1.2.4.2. AMR in the context of IK

Broad-spectrum topical antibiotic therapy is the gold standard treatment for IK. Depending on the disease severity and clinicians' preference, antibiotic therapy is commonly administered in the form of dual therapy using cephalosporin and aminoglycoside or monotherapy using fluoroquinolone (McDonald et al., 2014). As intensive topical antibiotics are applied directly and frequently during the treatment of IK, high concentration of antibiotics can be effectively achieved at the target site (i.e. the infected cornea), which can potentially reduce the risk of AMR in ocular infections. However, a few recent IK studies have highlighted the emergence of AMR in ocular infections, particularly in the US (Asbell et al., 2020), China (Lin et al., 2019), and India (Acharya et al., 2019). The driving force is likely to be multifactorial, including the injudicious widespread use of antibiotics in both ocular and systemic infections (Brown, 2007), incorrect dosing regimen (Martinez et al., 2012), and representations of the community prevalence of drug resistance, with consequent colonisation of OS by drug resistant pathogens (Blomquist, 2006). For instance, in the SCUT study, there was a 3.5-fold higher MIC for bacteria isolated from patients who had previous treatment with fluoroquinolones compared to treatment naive patients (Ray et al., 2013).

A tabulated summary of the literature concerning the *in vitro* antibiotic susceptibility and resistance of IK-related bacteria is provided in **Table 1.3** (Tam et al., 2017, Tan et al., 2017, Tavassoli et al., 2019, Ting et al., 2021h, Peng et al., 2018, Asbell et al., 2020, Vola et al., 2013, Hernandez-Camarena et al., 2015, Kaliamurthy et al., 2013,

Hsiao et al., 2016, Politis et al., 2016, Acharya et al., 2019, Lin et al., 2019, Cabrera-Aguas et al., 2019, Green et al., 2019a). Overall, fluoroquinolone-resistant, methicillin-resistant, and multidrug resistant (MDR; i.e. resistant to three or more antibiotics) infections are being increasingly reported in IK (Asbell et al., 2020, Hernandez-Camarena et al., 2015, Kaliamurthy et al., 2013, Acharya et al., 2019, Lin et al., 2019, Vola et al., 2013). Geographical and temporal factors play a role in the variation of AMR pattern in ocular infections. Reports from Southern India demonstrated that multidrug resistance (i.e. resistant to three or more antibiotics) was most commonly observed among *S. pneumoniae* (44%), *S. epidermidis* (14.8%), *S. aureus* (14%), and *P. aeruginosa* (6%) (Kaliamurthy et al., 2013). However, gatifloxacin – a fourth-generation fluoroquinolone – was effective against the majority of Gram-negative bacteria (~90%), including *P. aeruginosa* and *Acinetobacter spp.,* thus its use as a monotherapy in Gram-negative IK was recommended in that region (Kaliamurthy et al., 2013). Another study from Southern China similarly reported an increase in MDR among Gram-positive cocci to several antibiotics from 2010 to 2018, while susceptibility to fluoroquinolone and aminoglycoside among Gram-negative bacilli remained stable (Lin et al., 2019). In contrast, a Northern India study reported a high rate of resistance of *P. aeruginosa* against ciprofloxacin (57%), moxifloxacin (47%), and aminoglycoside (52-60%) (Acharya et al., 2019), highlighting the geographical disparity in the AMR pattern and the importance of region-specific interrogation of the AMR profile in ocular infections.

An increasing trend in MRSA-related ocular infection has also been reported in several studies in the past decade. The Antibiotic Resistance Among Ocular Microorganisms (ARMOR) study in the US observed that a high rate of AMR, specifically methicillin resistance, was observed among *Staphylococci spp.* and *Streptococci spp.* and the risk increased with age (Asbell et al., 2020). More worryingly, ~75% of the MRSA and MR-CoNS were MDR. Another US study

demonstrated an increased rate of MRSA-related IK as well as resistance against fluoroquinolones, which questioned their ongoing use as primary monotherapy (Peng et al., 2018). Similarly, a 10-year Mexico study showed that 21-79% of the *S. aureus* and 48-71% of the CoNS were resistant to oxacillin (or methicillin). *P. aeruginosa* and other Gram-negative infections displayed resistance against oxacillin (86% and 90%, respectively) and vancomycin (97% and 70%, respectively), with an increasing trend of resistance to ceftazidime over time (Hernandez-Camarena et al., 2015). Another study conducted in Taiwan also highlighted the emerging issue of methicillin resistance, with MRSA accounting for 43% of all Gram-positive IK (Hsiao et al., 2016). On the other hand, an increase in voriconazole resistance was observed in the Mycotic Ulcer Treatment Trial (MUTT)-I for fungal keratitis, with a 2.1-fold increase in the mean MIC per year after adjustment for causative organism (Prajna et al., 2016).

Reassuringly, reports from the UK showed that Gram-positive bacteria exhibited a high susceptibility to cephalosporin (87-100%), but a moderate susceptibility to fluoroquinolone (61-81%) (Tan et al., 2017, Tavassoli et al., 2019, Ting et al., 2021b). However, Gram-negative bacteria were highly susceptible to both aminoglycoside (97-100%) and fluoroquinolone (91-100%) (Tan et al., 2017, Tavassoli et al., 2019, Ting et al., 2021h), suggesting that current antibiotic regimen (fluoroquinolone monotherapy or cephalosporin-aminoglycoside dual therapy) can safely remain as the first-line treatment in the UK. In the recent 12-year Nottingham Infectious Keratitis Study, an increasing trend of resistance against penicillin over time in both Gram-positive and Gram-negative isolates was observed, but a generally good susceptibility to aminoglycosides and fluoroquinolones was maintained; therefore, no change of antibiotic regimen was required (Ting et al., 2021h).

Table 1.3. A summary of the in vitro antimicrobial susceptibility and resistance of the causative microorganisms of infectious keratitis.

Year	Authors	Study period	Region	No. of cases	Antibiotic susceptibility (%)*		
					CEP	AMG	FQ
UK and Europe							
2017	Tan et al.	2004-2015	UK	4229	86 (P); 61 (N);	88 (P); 97 (N)	83 (P); 91 (N)
2019	Tavassoli et al.	2006-2017	UK	2614	-	100 (P); 97.0-100 (N)	91-100 (P); 97-100 (N)
2021	Ting et al.	2007-2019	UK	1333	100 (P); 81 (N)	95 (P); 98-99 (N)	90-100 (P); 98-100 (N)
North America							
2017	Tam et al.	2000-2015	Canada	2330	-	96 (P)	96 (P)
2018	Peng et al.	1996-2015	US	2203	-	50-100 (N)	85-100 (P); 80-100 (N)
2020	Asbell et al.	2009-2018	US	6091	-	97 (MSSA); 62 (MRSA); 94 (MS-CoNS); 71 (MR-CoNS); 97 (N)	89-90 (MSSA); 26-29 (MRSA); 88-89 (MS-CoNS); 43-49 (MR-CoNS); 93-100 (N)
South America							
2013	Vola et al.	2000-2009	Brazil	566	-	93 (MSSA); 70 (MRSA)	96 (MSSA); 62 (MRSA)
2015	Hernandez-Camarena et al.	2002-2011	Mexico	1638	18-90 (P); 10-92 (N)	42-80 (P); 69-98 (N)	54-100 (P); 87-100 (N)
Asia							
2013	Kaliamurthy et al.	2005-2012	India	2170	-	31-95 (P); 90-93 (N)	70.4-98 (P); 74-90 (N)
2016	Hsiao et al.	2003-2012	Taiwan	2012	-	85-88 (N)	89 (P); 94 (N)
2019	Acharya et al.	2015-2017	India	1169	-	73 (P); 89 (N)	69 (P); 69 (N)
2019	Lin et al.	2010-2018	China	7229	84-91 (P); 68-75 (N)	-	63-75 (P); 46-75 (N)
Africa and Middle East							
2016	Politis et al.	2002-2014	Jerusalem	943	-	92-94 (P)	97-100% (P)
Australasia							
2019	Cabrera-Aguas et al.	2012-2016	Australia	1084	-	86-97 (P); 100 (N)	86-95 (P); 99 (N)
2019	Green et al.	2005-2015	Australia	3182	-	92 (P); 96 (N)	94 (P); 99 (N)

MSSA = Methicillin-sensitive *Staphylococcus aureus*; MRSA = Methicillin-resistant *S. aureus*; MS-CoNS = Methicillin-sensitive coagulase negative staphylococci; MR-CoNS = Methicillin-resistant coagulase negative staphylococci

*Antibiotic susceptibility is reported for Gram-positive bacteria (P) and Gram-negative bacteria (N) against three common classes of antibiotics, namely cephalosporin (CEP), aminoglycoside (AMG), and fluoroquinolone (FQ).

1.2.4.3. Clinical impact

AMR represents a global challenge with a huge impact on morbidity and mortality. It was estimated that 2 million people / year in the US are infected with antimicrobial resistant organisms, with a $20 billion cost incurred on the healthcare system. A recent UK report predicted a global loss of $100 trillion by 2050 related to AMR (O'Neill, 2016).

Within the context of IK, AMR was found to negatively affect the clinical outcome of IK. Kaye et al. (2010) observed that the corneal healing time of IK was prolonged with the increase of minimum inhibitory concentration (MIC; i.e. antibiotic resistance) of the causative organisms, including *P. aeruginosa*, *S. aureus* and *Enterobacteriaceae spp.*, against fluoroquinolone monotherapy. In addition, Lalitha et al. (2012a) demonstrated that higher level of MIC was associated with a significantly increased risk of corneal perforation in fungal keratitis.

AMR is continuing to increase in an alarming way. There is a pressing need to increase the awareness amongst prescribers on judicious use of antimicrobials, to tighten the control of over-the-counter antimicrobials in many countries, and to develop novel therapeutic modalities and strategies for IK, including therapeutic CXL and host defence peptides (or previously known as antimicrobial peptides), which hold great promises as a new class of antimicrobials in the future (Ting et al., 2020a, Mayandi et al., 2020, Mohammed et al., 2019, Ting et al., 2019e).

1.2.5. Medical management

1.2.5.1. Topical treatment

Broad-spectrum topical antimicrobial treatment represents the mainstay of treatment for treating IK. Depending on the disease severity, clinicians' preference and region-specific microbiological profiles, fluoroquinolone monotherapy (e.g. levofloxacin) and

dual therapy, consisting of a cephalosporine (e.g. cefuroxime) and aminoglycoside (e.g. amikacin), are the most common choices of antimicrobial agents (McDonald et al., 2014, Ting et al., 2021a). In regions where there is a relatively higher rate of MRSA-related IK, topical vancomycin instead of topical cephalosporin will be the preferred choice (Austin et al., 2017b). Common topical antifungal treatment includes amphotericin, natamycin and voriconazole whereas topical anti-acanthamoebic treatment includes biguanide (e.g. polyhexamethylene biguanide (PHMB) and chlorhexidine) and diamidines (e.g. propamidine and hexamidine) (Austin et al., 2017a). In view of excessive tearing during infection, blinking and tear drainage, topical antimicrobial treatment is commonly administered hourly during the initial phase to achieve the desired therapeutic concentration at the ocular surface, followed by a tapering course of treatment subject to satisfactory clinical improvement.

1.2.5.2. Subconjunctival / intrastromal injection

In refractory or severe cases, other modes of antimicrobial drug delivery such as subconjunctival and intrastromal injections can be given (Tsai et al., 2016). The rationale of these injections is to create a reservoir of antimicrobial drugs which will allow for a more sustained release of treatment at the ocular surface. Such mode of treatment also obviates the issue of excess tearing, which can wash out the administered topical antimicrobial treatment. Aydin et al. (2020) demonstrated that combined intrastromal amphotericin B and voriconazole serve as an effective adjuvant treatment to topical antifungal treatment for managing persistent fungal keratitis. Similarly, in a 10-year fungal keratitis study in the UK, Ting et al.(2021d) highlighted that adjuvant intrastromal voriconazole served as a useful adjuvant treatment for managing refractory fungal keratitis that did not respond to intensive topical antifungal treatment. However, in a randomised controlled trial of 70 eyes, the study did not demonstrate any additional benefit of intrastromal voriconazole for treating severe filamentous fungal keratitis compared to topical natamycin alone

(Narayana et al., 2019b), suggesting that the efficacy of intrastromal injection may be dependent on the type of antifungal agent and the underlying causative organism.

1.2.5.3. Systemic treatment

Although uncommonly used, oral antimicrobial treatment may provide additional therapeutic effect for IK, particularly those that involve the limbus or the sclera (i.e. sclerokeratitis). In addition, the Mycotic Ulcer Treatment Trial (MUTT)-2 has shown that adjuvant oral voriconazole could reduce the risk of severe complications such as corneal perforation in patients with severe Fusarium keratitis (Prajna et al., 2017). Recently, oral miltefosine, an anti-Leishmaniasis drug, has been shown to be effective against refractory Acanthamoeba keratitis (Thulasi et al., 2021).

1.2.6. Surgical management

1.2.6.1. Therapeutic corneal cross-linking

Corneal collagen cross-linking (CXL) was first introduced in 2003 by Wollensak et al.(2003) to stabilise the progression of keratoconus. It utilises a combination of ultraviolet-A (UVA) light of 370 nm and photosensitising agent “riboflavin” to increase the corneal biomechanical stability and rigidity. The long-term efficacy and safety of CXL for corneal ectatic disorders have been well established by many long-term studies (Caporossi et al., 2010, Hashemi et al., 2013, Mazzotta et al., 2018, Ting et al., 2019f). In addition to the stiffening effect on the cornea, CXL has been increasingly used for IK in the recent years. The rationale for using CXL for infection is based on the strong inherent antimicrobial activity of the UV light, which can directly damage the DNA and RNA of various types of microorganisms. Furthermore, the reactive oxygen species released from photoactivated riboflavin can directly affect the DNA and cell membranes of the microorganisms, culminating in a powerful synergistic antimicrobial action (Kumar et al., 2004, Naseem et al., 1988, Tsugita et al., 1965). These effects together with the increased corneal rigidity and hence

resistance to proteolytic enzymatic digestion of stromal collagen has made CXL an attractive adjuvant in the management of IK (Garg et al., 2017a).

In view of the emerging evidence of CXL for infectious keratitis, a new terminology – Photo-Activated Chromophore for Keratitis – Corneal Collagen Cross-Linking (PACK-CXL) – was coined in 2013 at the ninth CXL congress in Dublin, Ireland, to help distinguish its use from CXL for corneal ectasia and to avoid scientific confusion (Hafezi and Randleman, 2014). However, PACK-CXL is not routinely used in clinical practice due to the uncertainty of its efficacy and safety. This is primarily attributed to the wide heterogeneity of the literature in relation to the patient cohort, causative microorganisms, characteristics and severity of the ulcers, and treatment protocol, and the lack of large randomised control trials (RCTs).

1.2.6.2. Amniotic membrane transplantation

Amniotic membrane (AM) is the innermost layer of the fetal membrane, comprising a monolayer of metabolically active epithelium, a thick basement membrane, and an avascular stromal matrix (van Herendael et al., 1978). AM has been shown to possess a plethora of beneficial biological properties, including anti-inflammatory, anti-angiogenic, antimicrobial, wound healing, and anti-fibrotic functions (Dua et al., 2004). In view of the multi-faceted functions, easy accessibility to AM donor tissues, refinement of preservation and storage methods, and lack of donor tissue-related immunogenicity, AMT is now widely deployed as part of the management of many ocular surface diseases, including persistent epithelial defect / non-healing corneal ulcer, IK, corneal perforation, chemical eye injury, limbal stem cell deficiency, cicatricial conjunctivitis, radiation keratopathy, symptomatic bullous keratopathy and pterygium surgery, amongst others (Jirsova and Jones, 2017, Dua et al., 2020, Dua et al., 2017, Dua et al., 2018, Maharajan et al., 2007, Paris Fdos et al., 2013, Ting et al., 2021n, Dua et al., 2021, Ahmmed et al., 2021).

To date, a number of studies have evaluated the benefit of AMT for treating active IK, though the majority of them were of small case series or case reports. In clinical practice, AMT is usually reserved as a second-line therapy in IK, mainly to promote cornea healing in non-healing ulcer after the sterilisation phase. Therefore, the value of employing AMT in addition to standard antimicrobial treatment (SAT) during the active phase of IK remains uncertain.

1.2.6.3. Conjunctival hooding

Conjunctival hooding, or also known as Gunderson flap, is a surgical technique that involves covering an area of corneal defect with a conjunctival graft / pedicle. While uncommon, this technique can be employed to manage persistent epithelial defect, severe infection and threatened / actual corneal perforation (usually with guarded visual prognosis) (Ting et al., 2021a, Trinh et al., 2021). Nizeyimana et al.(2017) demonstrated that conjunctival hooding serve as a useful alternative surgery for managing refractory fungal keratitis, particularly in countries where donor corneas are lacking for keratoplasty.

1.2.6.4. Therapeutic / tectonic keratoplasty

Over the past few decades, the field of corneal transplantation has undergone rapid evolution, primarily attributed to the improved understanding of immunological rejection, innovation in surgical instrumentation and technique, and advancement in cell therapy (Tan et al., 2012, Ting et al., 2012, Kinoshita et al., 2018, Ting et al., 2021m). However, the success of keratoplasty is limited by the global shortage of donor corneas (Gain et al., 2016), for which important measures such as improving public awareness of eye donation, introduction of telephone consent, opt-out donation system, improvement of eye bank systems, and cornea bioengineering have been

considered and implemented (Gopal et al., 2021, Gupta et al., 2018, Ting et al., 2016a, b, Ting et al., 2021b).

Indications for keratoplasty can be broadly divided into four categories, namely optical, therapeutic, tectonic and cosmetic keratoplasty. Therapeutic keratoplasty is performed to treat severe and/or refractory corneal infection or disease by replacement of the damaged host cornea with a healthy cornea whereas tectonic keratoplasty is indicated in cases with actual or threatened corneal perforation to preserve the anatomical integrity of the globe (Said et al., 2021). The terms therapeutic and tectonic are at times used interchangeably and often there is an overlap between categories as more than one purpose is served by the procedure. Therapeutic and tectonic keratoplasty serve as important eye-saving surgeries to maintain the globe integrity and/or treat recalcitrant corneal infection (Hossain et al., 2018, Ting et al., 2019c, Ting et al., 2021d). However, studies have shown that therapeutic / tectonic keratoplasty in “hot eyes” usually carries a worse prognosis than those performed for optical purpose (Khor et al., 2018, Ting et al., 2021d).

1.3. Host defense peptides (HDPs)

Being constantly exposed to pathogens, environmental irritants and stress, the OS relies on a highly functional innate immunity. Innate immunity mechanisms for the OS are composed of three major components, including the physical barrier (e.g. epithelial layers of conjunctiva, cornea and adjacent epidermis, blinking reflex, and others), chemical barriers (e.g. presence of antimicrobial components in tears) and cellular responses (e.g. macrophages, neutrophils, and others), for which the host defense peptides play important roles in the latter two.

Antimicrobial peptides (AMPs) are a group of evolutionarily conserved molecules of the innate immunity (Hancock and Lehrer, 1998). To better capture the increasingly recognised multi-faceted roles of AMPs, a broader term “host defense peptides (HDPs)” has been subsequently introduced (Haney et al., 2019). They are ubiquitously expressed at epithelial surfaces (e.g. eye, skin, respiratory, gastrointestinal linings, etc.) and secreted by immune cells (e.g. polymorphonuclear leukocytes and macrophages) (Mansour et al., 2014, Mohammed et al., 2017). So far more than 3000 naturally occurring and synthetic HDPs have been discovered across six life kingdoms (Zhao et al., 2013, Wang et al., 2016). These HDPs are usually cationic (due to the relative excess of arginine and lysine residues) and amphiphilic, with 30-50% hydrophobicity. They have recently shown promise as potential therapeutic agents due to their broad-spectrum antimicrobial properties against a wide array of infection, including drug-resistant bacteria, fungi, Acanthamoeba, and viruses, with minimal risk of developing AMR. In principle, HDPs are shown to primarily exert their broad-spectrum and rapid antimicrobial action through three main mechanisms of action, namely the barrel-stave, toroidal and carpet models (Li et al., 2017, Bechinger, 2011, Mookherjee et al., 2020) (**Figure 1.3**). The positively charged amino acid residues are responsible for the adsorption of AMPs onto the anionic bacterial membrane (via electrostatic interaction) and the hydrophobic residues interact with the lipid tail region of the membrane, culminating in membrane permeation, leakage of fluid into the bacterial cytoplasm and subsequent bacterial cell death (Li et al., 2017). In addition to the membrane-targeting action, emerging evidence has highlighted that HDPs can kill microorganisms through several non-membrane perturbing mechanisms, such as biosynthesis of disorganised bacterial membranes and direct intercalation into the membrane, interfering with the intracellular DNA and RNA molecules, and others (Haney et al., 2019). HDPs are also shown to participate in chemotaxis, immunomodulation, wound healing, anti-biofilm and anti-cancer activities (Steinstraesser et al., 2008, Hancock et al., 2016, Pletzer

et al., 2016, Riedl et al., 2011), offering a wide range of potential therapeutic applications.

The history of HDPs (or AMPs) dates back to 1922 when lysozyme was first discovered in various human tissues and body secretions, including the tear fluids (Fleming, 1922). Since then, a wide spectrum of human HDPs have been identified and reported at the OS. Haynes et al. (1998) were the first to scrutinise and report the expression of alpha- and beta-defensins at the OS, particularly in tears, conjunctiva, cornea and lacrimal gland. Since then, a number of HDPs, including lactoferrin, human alpha- and beta-defensins, human cathelicidin (LL-37), ribonuclease, psoriasin and dermcidin, amongst others, have been discovered at the OS (Abedin et al., 2008, Dua et al., 2014, Haynes et al., 1999, McIntosh et al., 2005, Mohammed et al., 2017, Otri et al., 2012b, Gordon et al., 2005, Huang et al., 2007a, Kolar and McDermott, 2011). Many of these HDPs are constitutively expressed and readily inducible during inflammation and infection of the OS, highlighting their role in the innate immunity of OS.

1.3.1. Spectrum and characterisation of HDPs at ocular surface

A wide array of HDPs have been identified and reported at the OS. In this section, the sources, characteristics, and functions of important HDPs, including lysozyme, lactoferrin, alpha- and beta-defensins, cathelicidin, ribonucleases, psoriasin, and dermcidin are summarised in **Table 1.4**.

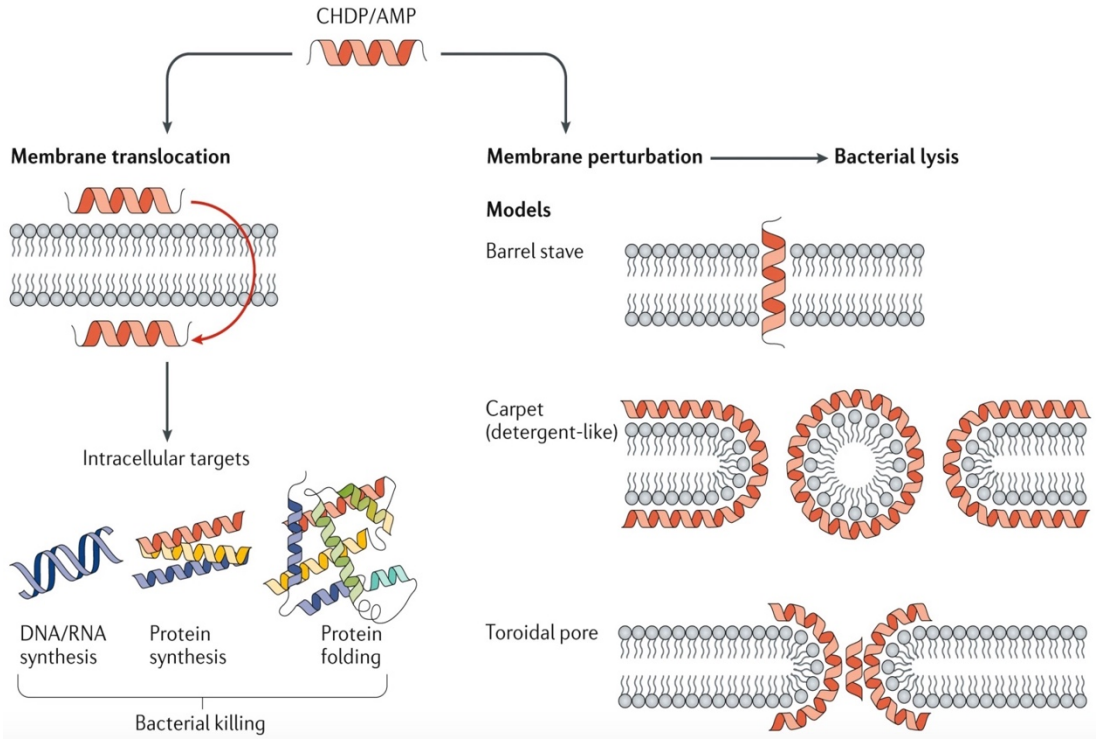


Figure 1.4. Mechanisms of action of host defense peptides (HDPs).
CHDP = Cationic host defense peptide; AMP = Antimicrobial peptide

This figure was reproduced from the paper published by Mookherjee et al. (2020) with permission.

1.3.1.1. Lysozyme

In 1922, lysozyme was discovered by Sir Alexander Fleming during the investigation of his patient with acute coryza. The nasal secretion of this patient was found to completely inhibit the growth of *Micrococcus spp.* (a Gram-positive bacteria). This striking observation prompted a series of experiments, which led to the discovery of lysozyme in various human tissues and body secretions, including tear fluids, saliva, blood, semen, respiratory tract linings, and connective tissues, amongst others (Fleming, 1922). Interestingly, the antibacterial potency of lysozyme was influenced by the location of the tissues and types of microbes (e.g. lysozyme in tears was very active against micrococci, but was much less effective against other cocci in other parts of the body), highlighting the specific adaptation of the human immune system against specific pathogens at defined sties (Fleming, 1922).

Lysozyme is primarily secreted in the tear fluid by the tubuloacinar cells of the main and accessory lacrimal glands (McDermott, 2013) and, to a lesser extent, expressed by corneal epithelium and meibomian glands (Tsai et al., 2006). It constitutes around 20-30% of the total protein in tear fluids (McDermott, 2013). Lysozyme exhibits its broad-spectrum antimicrobial activity via dual mechanisms of action (Ragland and Criss, 2017). First, it hydrolyses the bacterial cell wall by breaking down the β-1,4 glycosidic linkages between the disaccharides, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), which forms the backbone of peptidoglycan in the bacterial membrane. Second, the cationic property of lysozyme enables pore formation in the anionic bacterial membrane, which is responsible for its rapid and broad-spectrum antimicrobial activity against a wide range of organisms.

In addition to its antimicrobial activity, lysozyme plays an important immunomodulatory role in host defense. Particularly, it activates lysozyme-dependent degradation of the engulfed bacteria within the phagolysosomes of

macrophages and releases pathogen associated molecular patterns from the lysed bacteria, resulting in a pro-inflammatory response via interaction with various pattern recognition receptors such as Toll-like receptors (TLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs), and inflammasomes (Ragland and Criss, 2017). Lysozyme may decrease systemic inflammation by restricting bacterial growth (Ganz et al., 2003). In view of the ubiquitous presence and inherent antimicrobial and immunomodulatory activities of host lysozyme, bacteria have evolved several ingenious resistant mechanisms to survive against lysozyme. These include modification of membrane peptidoglycan, alteration of the membrane charges, and production of protein inhibitors against lysozyme (Ragland and Criss, 2017). The understanding of the mechanisms of AMR related to lysozyme (and potentially other naturally occurring HDPs) is pivotal for development of the next generation of synthetic HDP-based therapeutics for tackling AMR.

1.3.1.2. Lactoferrin

Lactoferrin, belongs to the transferrin family, is an 80 kDa iron-sequestering HDP. It consists of a polypeptide chain that is folded into two highly symmetrical lobes (N- and C-lobes), which are capable of binding a variety of metal ions including ferric and ferrous ions (Garcia-Montoya et al., 2012). It is found abundantly in milk and in many other body tissues and secretions, including tears, saliva, sweat, nasal secretion, bronchial mucus, hepatic bile and others (Hao et al., 2019). Similar to lysozyme, lactoferrin is also primarily synthesised by the acinar cells of the main and accessory lacrimal glands (Gillette and Allansmith, 1980). Some evidence has suggested the expression of lactoferrin in meibomian glands (Tsai et al., 2006) and epithelium of conjunctiva and cornea (Tsai et al., 2006, Santagati et al., 2005). It constitutes around 25% of the total protein in tear fluids, with a concentration of ~2.2 mg/ml (Flanagan and Willcox, 2009).

Table 1.4. Common HDPs at the ocular surface (OS) and skin.

Type	Source	Functions
Lysozyme	OS: Tear fluid (secreted by tubuloacinar cells of lacrimal glands), corneal epithelium, meibomian glands Skin: Cytoplasm of epidermal cells, eccrine sweat glands, pilosebaceous follicles and dermal collagen bundles	- Antimicrobial property (via hydrolysis and pore formation of cell wall) - Immunomodulatory function via interaction with various PRRs
Lactoferrin	OS: Tear fluid (secreted by acinar cells of lacrimal glands), conjunctival epithelium, corneal epithelium, and meibomian glands	- Antimicrobial activity (via binding to free iron and membrane permeabilization) and anti-biofilm - Anti-inflammatory - Antioxidant (via inhibition of iron-dependent formation of hydroxyl radicals) - Wound healing
Defensins	OS: (a) HNP1-4: Azurophil granules of neutrophils; (b) HBD: Conjunctival and corneal epithelium Skin: HNP1-4: Epidermis and/or keratinocytes;	- Antimicrobial activity (via membrane perturbation) - Immunomodulatory function (pro- and anti-inflammatory) - Wound healing - Anti-cancer
Cathelicidin	OS: Conjunctival epithelium, corneal epithelium Skin: Epidermis, keratinocytes (during infection or injury)	- Antimicrobial activity and anti-biofilm - Immunomodulatory function (pro- and anti-inflammatory) - Wound healing - Anti-cancer
RNases	OS: RNase-5: Tear fluid and corneal endothelium; RNase-7: Corneal epithelium and stroma Skin: RNase-5: Keratinocytes; RNase-7: Epidermis	- Antimicrobial activity - Immunomodulatory function (activates adaptive immunity) - Angiogenic and neurogenic - Wound healing
Psoriasin	OS: Conjunctiva, cornea, lacrimal gland, and nasolacrimal duct Skin: Epidermis (low level) and keratinocytes	- Antimicrobial activity (via zinc-dependent mechanism) - Immunomodulatory function (chemotaxis, activates adaptive immune system)
Dermcidin	OS: Corneal epithelium and tear fluid Skin: Eccrine sweat (from secretory granules)	- Antimicrobial activity (via zinc-dependent mechanism)

PRR = Pattern recognition receptors; HNP = Human neutrophil peptide / human alpha-defensin; HBD = Human beta-defensins

Lactoferrin has been shown to play multi-functional roles in host defense, armed with antimicrobial, anti-biofilm, anti-inflammatory, anti-cancer and anti-complement functions (Garcia-Montoya et al., 2012, Samuelsen et al., 2004). The antimicrobial activity of lactoferrin is attributed to its underlying dual mechanisms of action: (a) binding to free iron, an essential element for microbial growth; and (b) interaction and permeabilisation of the anionic bacterial membrane through its positively charged N-terminal, which accounts for its rapid antimicrobial action (Garcia-Montoya et al., 2012). At the OS, it has been shown to exert broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses (Flanagan and Willcox, 2009). It has a strong affinity toward the lipopolysaccharides (LPS) of the Gram-negative bacterial membrane, resulting in increased permeability. Studies have also shown that lysozyme and lactoferrin work in synergy where lactoferrin binds to the lipotechoic acid of staphylococcal membrane and enables a greater access of lysozyme to the peptidoglycan (Leitch and Willcox, 1999). Interestingly, lactoferrin is ineffective against Acanthamoeba trophozoites and this is attributed to the effect of proteases released by Acanthamoeba (Ramirez-Rico et al., 2015).

Lactoferrin also acts as an antioxidant via inhibition of iron-dependent formation of hydroxyl radicals, thereby protecting corneal epithelium from oxidation-mediated tissue injury (Kijlstra, 1990). Furthermore, reduced levels of lactoferrin have been associated with systemic mucosal immunity incompetence. Hanstock et al. (2019) observed that patients affected by upper respiratory tract infection had a significantly lower concentration of tear lysozyme and/or lactoferrin compared to healthy volunteers, suggesting that lysozyme and lactoferrin may serve as clinically relevant biomarkers for mucosal immune competence.

1.3.1.3. Defensins

Defensins are a large family of cysteine-rich HDPs that consist of a predominantly triple-stranded beta-sheet core structure stabilised with three pairs of intramolecular disulfide bridges (Ganz, 2003). Depending on the pattern of the disulfide linkage, human defensins can be broadly divided into two groups, namely the alpha- and beta-defensins. Alpha-defensins have a cysteine pairing motif of Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5 whereas beta-defensins form disulfide bridges at Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6 (Lehrer and Lu, 2012, Ganz, 2003). Interestingly, this evolutionarily conserved disulphide bridge motif is similarly observed in defensins found in plants and invertebrates (Stotz et al., 2009, Bulet et al., 2004).

Human alpha-defensins, also known as human neutrophil peptides (HNPs) due to their abundant presence in neutrophils, can be subclassified into 6 main subtypes (HNP-1 to -6). HNP-1 to -4 are found primarily in the azurophil granules of neutrophils (Lehrer and Lu, 2012). HNP-1 to -3 sequences are highly homologous with only difference in a single N-terminal residue; removal of the alanine (the first amino acid of HNP-1 at the N-terminal) gives rise to HNP-2 and substitution of the alanine with aspartic acid yields HNP-3. On the other hand, more than 30 types of human beta-defensins (HBDs) have been described in the literature (Pazgier et al., 2006). HBDs are mainly synthesised by the epithelial cells, including the conjunctiva, cornea, skin, oral mucosa, lining of respiratory and gastrointestinal tracts, and others (Weinberg et al., 2012). As described by McIntosh et al. (McIntosh et al., 2005), about 28 novel beta defensins were identified in human genome using the hidden Markov model. Thus far, only few, namely the HBD-1 to -4 and HBD-9 were shown to be involved in host immunity at the OS (Mohammed et al., 2017).

In view of the diverse function of defensins, it is not surprising that a plethora of HNPs and HBDs are abundantly present at a variety of bodily surfaces. At the OS, HNP-1

to -3 have been identified in normal human tears, conjunctival and corneal epithelium, lacrimal gland, and inflamed conjunctiva (in relation to infiltrating polymorphonuclear cells) (Haynes et al., 1999, 1998, Ikeda et al., 2005). Similarly, McIntosh et al. (2005) discovered an array of HBDs, including HBD-1 to -4, at the corneal and conjunctival epithelium, though the level of HBD-4 was relatively low. Another novel HDP, HBD-9, was discovered at the OS epithelia and corneal stroma by Dua's group (Mohammed et al., 2010, Abedin et al., 2008). However, possible antimicrobial functions of HBD-9 remain unknown. Further studies have also shown that the expressions of HBDs are modulated by various pattern recognition receptors, including TLRs and NLRs (Redfern et al., 2011, Mohammed et al., 2010, Redfern and McDermott, 2010). The expression of HBD-1 to -3 are upregulated in the event of infection and inflammation, albeit the mechanisms of induction/upregulation are different from each other (Harder et al., 1997, Sorensen et al., 2005).

Defensins have been shown to exhibit broad-spectrum antimicrobial activity against bacteria, fungi, enveloped viruses, and parasites (Semple and Dorin, 2012, Lehrer and Lu, 2012). Similar to most cationic HDPs, the defensins also perturb the microbial membrane through direct interaction with the anionic and lipid microbial membrane. The antimicrobial efficacy of defensins is likely related to their inherent physicochemical characteristics such as cationicity, hydrophobicity, and amphiphilicity (Lehrer and Lu, 2012). It has been shown that cationicity plays a more important role in Gram-negative infections, whereas increased hydrophobicity enhances the antimicrobial action against Gram-positive infections (Jiang et al., 2018). In addition, synergy between different families of HDPs have been reported; for instance, HBD-2 and LL-37 exhibit synergistic antimicrobial killing of *S. aureus* (Ong et al., 2002).

In addition to the antimicrobial function, defensins are endowed with a wide range of functions, including immunomodulatory (pro-inflammatory and anti-inflammatory), wound healing, and anti-cancer (Hancock et al., 2016, Semple and Dorin, 2012, Kiatsurayanon et al., 2018). HBD has been shown to orchestrate the crosstalk between innate and adaptive immunity by recruiting T cells and dendritic cells to the infection site through interaction with chemokine (CCR6) receptor (Yang et al., 1999). HNP-1 regulates inflammation by inhibiting macrophage mRNA translation and secretion of proinflammatory cytokines and nitric oxide, enabling clearance of pathogen and resolution of inflammation with minimal collateral tissue damage (Brook et al., 2016, Miles et al., 2009).

To gain a better understanding of the structure-activity relationship, many research groups have investigated the functional role of the evolutionarily conserved cysteine disulfide bridge moiety of defensins. Although this moiety is widely observed in vertebrate and invertebrate defensins, Wu et al. (2003) demonstrated that removal of this structure has no influence on their inherent antibacterial activity against *Escherichia coli.* On the other hand, the chemotactic function (e.g. HBD-3) (Wu et al., 2003), anti-tumour necrosis factor (TNF)-alpha (e.g. HNP-1) (Miles et al., 2009), and antiviral activity (e.g. HNP-1 to -3) (Daher et al., 1986) are abolished when this disulfide moiety is destabilised or removed, suggesting that the disulfide bridges play important immunomodulatory and antiviral roles in innate immunity. These observations provide invaluable insight into the design and development of antimicrobial HDPs that are based on cysteine-rich native templates (Lakshminarayanan et al., 2016).

1.3.1.4. Cathelicidin

Cathelicidins are a large family of AMPs widely found in vertebrates (Bulet et al., 2004, Coorens et al., 2017). The hallmark of cathelicidin is the presence of highly

conserved cathelin domain, which was first identified in pig leukocytes as a cathepsin-L inhibitor and termed 'cathelin' based on this property. Cathelicidin proteins comprised of a conserved 14 kDa cathelin domain flanked by a signal peptide (up to 30 residues) on N-terminus and an antimicrobial peptide region on its C-terminus. hCAP18, an 18 kDa preprotein, is the lone member of cathelicidin found in humans (Zanetti et al., 2002, Cowland et al., 1995). Its derivative, hCAP18(104-140), was shown to neutralise LPS activity both *in vitro* and *in vivo* (Larrick et al., 1995). Proteinase-3, a proteolytic enzyme in human neutrophils can cleave hCAP18 into an active 37 amino-acid AMP, known as LL-37 (Zanetti et al., 2002, Zasloff, 2019, Gudmundsson et al., 1996). Moreover, another serum protease, gastricsin, at low vaginal pH was shown to cleave hCAP18 into a slightly longer active peptide, termed ALL-38 (Sørensen et al., 2003). Since its first discovery in 1988 (Romeo et al., 1988), cathelicidin is the most studied cationic HDP due to its wide-spectrum of activity, including anti-infective, anti-biofilm, anti-cancer, immunomodulatory, chemotactic, and wound healing properties (Mookherjee and Hancock, 2007, Scheenstra et al., 2020, Ren et al., 2013, Ren et al., 2012).

The protective function of LL-37 against OS has been widely established (Huang et al., 2006, Huang et al., 2007b). LL-37 is constitutively expressed in OS epithelial specimens from healthy living patients and donor cadaveric tissues, including conjunctival and corneal epithelium (McIntosh et al., 2005, Gordon et al., 2005). It has been shown to play an important role in corneal wound healing and protection against various types of microbes at the OS (Huang et al., 2006, Gordon et al., 2005).

Biochemical studies have elegantly demonstrated that smaller synthetic fragments derived from the parent LL-37 sequence were as effective as the full-length LL-37 (Li et al., 2006, Wang et al., 2014, Engelberg and Landau, 2020, Wang, 2008b, Wang et al., 2012b). Studies have revealed that the middle region of LL-$37_{17\text{-}29}$ (i.e. FK13)

and/or LL-37_{18-29} (i.e. KR12) is largely responsible for the antimicrobial activity of LL-37 and has the ability to form amphipathic helix rich in positive charge, which enables effective interaction with the anionic membrane and subsequent microbial killing (Rajasekaran et al., 2017, Wang, 2008b, Engelberg and Landau, 2020). In view of its therapeutic promise, a variety of strategies have been adopted to enhance the safety and efficacy of LL-37 and its derivatives (Ting et al., 2020a, Ting et al., 2021e). Similar to LL-37, its smaller derivatives have shown considerable activity against a range of pathogens, including ESKAPE bacteria, fungi and viruses (Yu et al., 2020, Luo et al., 2017, Narayana et al., 2019a, He et al., 2018). Mohammed et al. (2019) recently demonstrated that LL-37_{17-32} (FK16 peptide with free N- and C-termini) could also be utilised to improve the activity of conventional antibiotics such as vancomycin against *P. aeruginosa*, as a strategy to repurpose the antibiotics and tackle AMR.

1.3.1.5. Ribonucleases

Human ribonucleases (RNases) have an inherent ability to hydrolyse polymeric RNA and share a unique structural similarity to bovine pancreatic RNase A, therefore, also referred to as RNase A superfamily (Rosenberg, 2008, Beintema et al., 1984). Similar to defensins, members of RNase A superfamily are comprised of 6 to 8 conserved cysteine residues forming disulfide bridges. Genes encoding for human RNases 1 to 13 are clustered on chromosome 14q11.2 (Raines, 1998, Beintema et al., 1984). RNases are highly cationic and exhibit strong cytotoxic and microbicidal properties. Human RNase-2 (eosinophil derived neurotoxin) and RNase-3 (eosinophil cationic protein) were the first members of RNase A superfamily to show a strong role in host defense against an RNA virus, respiratory syncytial virus (RSV) (Domachowske et al., 1998b, Domachowske et al., 1998a). Further studies have demonstrated that RNase-2 and -3 also have an ability to activate adaptive immunity (Yang et al., 2004, Yang et al., 2003) and possess potent bactericidal and anti-helminthic properties (Lehrer et al., 1989, Ackerman et al., 1983, Ackerman et al., 1985, Molina et al.,

1988). RNase-4 and -5 were shown to display potent angiogenic and neurogenic properties (Li et al., 2013a, Ferguson and Subramanian, 2018). RNase-5, also known as angiogenin, has been widely studied due to its immunomodulatory properties. It is shown to be produced by skin keratinocytes and mast cells and has been detected in lacrimal secretions. However, the function of RNase-4 and -5 in OS defense still remains unknown. RNase-6 is ubiquitously expressed in immune cells including neutrophils and monocytes. Similar to RNase-3, it also exhibits bactericidal effect through agglutination and membrane disruption (Lu et al., 2018). Against *Mycobacterium spp.*, it has been shown to induce autophagy in the infected macrophages leading to intracellular growth inhibition (Lu et al., 2019).

RNase-7 and -8 despite being structurally similar, their expression in different bodily tissues is greatly varied. On the OS, RNase-7 is constitutively expressed in healthy corneal epithelium and stroma (Mohammed et al., 2011). Further studies have demonstrated elevated levels of RNase-7 in samples collected from patients with bacterial, viral and Acanthamoeba keratitis as well as in corneal epithelial cells (CECs) treated with cytokines, live bacteria and different pathogenic proteins that activates innate immune receptor signalling (Mohammed et al., 2011, Otri et al., 2010). Specifically, the signalling mechanisms that are involved in elevation of RNase-7 levels in CECs in response to activation of interleukin 1β (IL-1β)/IL-1 receptor (IL-1R) axis was mapped by Mohammed et al. (2011). Notably, the canonical nuclear factor κB (NFκB) transcription factor which mediates transcription of most HDPs in OS epithelium was found to be non-redundant in regulation of RNase-7. It was shown that IL-1b/IL-1R triggered mitogen activated protein kinases (MAPKs) signalling was responsible for RNase-7 regulation in CECs. Further analysis showed that the transcription factors, c-JUN and ATF, are involved in transcription of RNase-

7 in CECs. This suggested that a biomarker or protein that directly activates these transcription factors could elicit HDPs in CECs during infection.

1.3.1.6. Psoriasin

Psoriasin, or S100A7, represents one of the main members of the S100 family of calcium-binding proteins (Donato, 1999). It is a low molecular weight protein (~11 kDa) which consists of five alpha-helices and the structure relies on binding of calcium (Brodersen et al., 1998). The term “psoriasin” was first coined in 1991 by Madsen et al., who observed the upregulation of this novel HDP in psoriatic skin (Madsen et al., 1991). Subsequently, its immunomodulatory role in psoriasis was shown to be related to the downstream stimulation of interleukin-1a (IL-1a) expression in human epidermal keratinocytes via the receptor for advanced glycation endproducts (RAGE)-p38 MAPK and calpain-1 pathways (Lei et al., 2017). Psoriasin was also found to be constitutively present in healthy skin (at a low concentration) and at the OS, including the conjunctiva, cornea, lacrimal gland and nasolacrimal ducts (Gläser et al., 2005, Garreis et al., 2011), highlighting its protective role at the skin and OS.

Psoriasin has been shown to exhibit strong antimicrobial activity against *E. coli* and *S. aureus*, likely via a zinc-dependent mechanism (Gläser et al., 2005, Lee and Eckert, 2007). The upregulation of psoriasin against *E. coli* was found to be mediated via TLR5 (Abtin et al., 2008). Interestingly, studies have shown that the antibacterial efficacy of psoriasin is likely conferred by the central region of the protein (amino acids at 35-80) (Lee and Eckert, 2007). In addition to its antimicrobial activity, psoriasin has been shown to play essential important immunomodulatory roles, including chemotaxis for CD4+ and neutrophils, production of cytokines and chemokines by neutrophils, generation of reactive oxygen species, and release of HNP-1 to -3 (Jinquan et al., 1996, Zheng et al., 2008).

1.3.2. Roles of HDPs in infectious keratitis

The pivotal roles of HDPs in IK are supported by a number of *in vitro* and *in vivo* observations and experiments (Mohammed et al., 2017, Huang et al., 2007a, Huang et al., 2006). McIntosh et al. (2005) investigated differential gene expression of HDPs in non-infected and infected eyes and demonstrated that some HDPs, notably HBD-3 and LL-37, were significantly elevated during OS infection. In addition, HBD-2 and -3, LL-37, MIP-3α, and thymosin β-4 were shown to exhibit moderate to strong *in vitro* antimicrobial activity against a range of ocular pathogens, including *S. aureus*, *P. aeruginosa*, adenovirus and HSV-1 (Huang et al., 2007a, Gordon et al., 2005). Furthermore, LL-37 deficient knockout mice were found to be more susceptible to *P. aeruginosa* corneal infection when compared to the wild type mice, underlining the antimicrobial function of LL-37 at the OS (Huang et al., 2007b). Synergistic antimicrobial action among different HDPs has also been reported in several studies (Chen et al., 2005, Singh et al., 2000). For instance, Chen et al. (2005) demonstrated that various combinations of skin-derived HDPs, including HBD-1 to -3, LL-37 and lysozyme, exhibited synergistic or additive antimicrobial effect against *S. aureus* and *E. coli*.

The role of HDPs has also been implicated in other types of IK such as fungal keratitis and AK (Mohammed et al., 2017). A recent study demonstrated that a range of HDPs, including HBD-1, -2, -3 and -9, LL-37 and S100A7, were upregulated during the active phase of fungal keratitis and returned to the baseline level upon resolution of the infection (Mohammed et al., 2020). Interestingly, there was a preferential increase in mRNA expression of different types of HDPs, with HBD-1 and -2 being most commonly upregulated (90% of the cases) and LL-37 being least commonly upregulated (35% of the cases), highlighting the pathogen-specificity of HDPs. Similarly in AK, a wide range of HDPs such as HBD-2 and -3, LL-37, liver-expressed antimicrobial peptide (LEAP)-1 and -2, and RNase-7 (but not HBD-1), were shown to

be upregulated (Otri et al., 2010). Interestingly, HBD-1 and HBD-9 were significantly downregulated in AK (Otri et al., 2012b, Otri et al., 2010). Taken together, it is evident that HDPs serve as an integral component of the innate immunity of the OS, via their broad-spectrum antimicrobial activity against a wide range of ocular pathogens. This unique characteristic also renders them an attractive class of antimicrobial agent for managing IK, particularly in the face of polymicrobial keratitis and emerging AMR (Ting et al., 2021j, Ting et al., 2019c, Asbell et al., 2020).

1.3.3. Strategies for enhancing antimicrobial efficacy and safety of HDPs

Despite their promising potential as effective antimicrobial and immunomodulatory therapies, several issues have impeded the successful translation of HDPs into clinical use. Complex structure-activity relationship (SAR), susceptibility to host / bacterial proteases and physiological conditions, pro-inflammatory properties, discrepancy between *in vitro* and *in vivo* efficacy, and toxicity to the host tissues are the main barriers (Kolar and McDermott, 2011, Hancock and Sahl, 2006, Fjell et al., 2011, Li et al., 2017, Ting et al., 2020a). Furthermore the dwindling interest and investment from the pharmaceutical companies, stemming from limited life-span of antimicrobial therapy and low profits, poses another significant hurdle for the development of new antimicrobial agent (Christoffersen, 2006).

Structurally, HDPs are mainly characterised by the presence of key charged residues (e.g. Arg and Lys), with a high proportion of hydrophobic residues (constituting 50% or more) and amphipathicity (Haney et al., 2017, Haney et al., 2019). To translate the therapeutic potential of HDPs, a number of strategies have been proposed and performed (Haney and Hancock, 2013, Haney et al., 2017, Ting et al., 2020a). In this

section, several key strategies to improve the selectivity of HDPs toward microbial membranes are summarized in **Table 1.5**,

1.3.3.1. Residue substitution with natural amino acids

HDPs should fulfil key functional requirements to qualify for clinical use, including low toxicity, high antimicrobial activity, and good *in vivo* stability (Fjell et al., 2011, Ting et al., 2020a). These requirements are closely linked to their biochemical selectivity toward anionic and zwitterionic surfaces (Fjell et al., 2011). The antimicrobial activity is attributed to a fine balance of hydrophobicity, cationic residues, amphipathicity and structural conformation (e.g. α-helical, β-sheet, and cyclised) (Raheem and Straus, 2019). On the other hand, the hydrophobic interaction between specific residues of HDPs (e.g. Leu, Ile, Val, Phe, Tyr, Trp) and zwitterionic phospholipids on host cell surfaces is responsible for its toxicity. For example, peptide derivatives of mastoparan (a key constituent of wasp venom) that were designed based on fixed five rules utilising the quantitative structure-activity relationship (QSAR) approach showed potent antimicrobial efficacy against *Bacillus subtilis* (Avram et al., 2012). It was shown that the potency of these derivatives was mainly dependent on the presence of Trp, Lys, and His (Avram et al., 2012). Lata et al. (2007) analysed 486 HDPs from the antimicrobial peptide database (APD) for amino acid frequency in these sequences using the bioinformatic tools. Residues such as Gly, Arg, Lys, and Leu were shown to be commonly found in HDPs, whilst AAs such as Ser, Pro, Glu, and Asp were least common at both N- and C-terminus. A recent study from Hilpert group has demonstrated through *in silico* designed library of 3000 *de novo* short peptides (9-mer in length) that the specific design characteristics of HDPs did not apply to short peptides (Mikut et al., 2016). The peptide sequences that were grouped as 'super active' based on their activity toward *P. aeruginosa* were mainly composed of Lys,

Arg, Trp, and Val/Ile/Leu (Mikut et al., 2016). However, the activity of these super peptides toward host cells and *in vivo* stability was not yet reported.

Melittin, a key constituent of honey-bee venom, is a potent HDP with strong antimicrobial activity (Memariani et al., 2019). However, its clinical use is largely limited by the high haemolytic activity (Memariani et al., 2019). Blondelle et al. (Blondelle et al., 1993) studied the function of Trp in melittin activity through serial Trp substitution starting from N- to C-terminus. Substitution of Leu→Trp at 9th position was shown to decrease the haemolytic activity whereas substitution of Pro→Trp at 14th position improved the alpha-helical conformation and reduced the haemolytic effects compared to parent melittin peptide.

HDPs with Pro residues are widely known to display a disrupted helix conformation, which eventually affects their surface retention time and penetration into microbe cytoplasm (O'Neil and DeGrado, 1990, Creamer and Rose, 1992). A recent study using peptide analogue (Anal 3, 19-AA long) from N-terminus of *Helicobacter pylori* ribosomal protein L1 has demonstrated that an insertion of Pro-hinge into Anal 3 (via Glu→Pro substitution at 9th position) significantly improves the peptide selectivity toward microbes with no effect on host cells (Lee et al., 2013, Putsep et al., 1999). This was attributed to the helix-hinge-helix conformation of Anal 3-Pro analogue at the surface of bacteria allowing peptide penetration and DNA binding in the cytoplasm. This study suggested that rational insertion of Pro residue through SAR analysis could improve the biological membrane selectivity of microbicidal peptides. Proline-rich designed HDPs such as ARV-1502 (Brakel et al., 2019), oncocin (Knappe et al., 2010), and Bac-5 (Mardirossian et al., 2019a, Mardirossian et al., 2019b) have shown significant efficacy against Gram-negative pathogens but not host cells membranes. Unlike cationic HDPs, proline-rich peptides kill bacteria through inhibition of protein synthesis (Mardirossian et al., 2019b, Mardirossian et al., 2018,

Graf et al., 2017, Cardoso et al., 2019a). Histidine-rich (Mason et al., 2006, Mason et al., 2009), alanine-rich (Migliolo et al., 2012), and tryptophan-rich (Deslouches et al., 2013) short HDPs have also been developed. These were shown to be highly effective at acidic pH against a range of Gram-negative and Gram-positive bacteria (Mishra et al., 2018).

Magainin-2 is 23 residues long AMP isolated from frog skin, *Xenopus laevis* (Zasloff, 1987). Due to its non-haemolytic and broad-spectrum antimicrobial properties, magainin-2 was widely studied as a model peptide to understand the SAR of naturally occurring AMPs (Zasloff et al., 1988, Esmaili and Shahlaei, 2015, Nguyen et al., 2009, Westerhoff et al., 1989, Maloy and Kari, 1995). Chen et al. (1988) have demonstrated that the alpha-helical conformation of magainin-2 could be stabilised through Gly→Ala substitution (at both 13^{th} and 18^{th} position) and C-terminal amidation. This was shown to increase the antibacterial activity by two-fold against a range of bacteria without modulating its safety against erythrocytes (Chen et al., 1988). Numerous groups have made attempts to improve the activity of magainin-2 against Gram-negative bacteria through residue substitution. It was demonstrated that the substitution of Phe→Trp in magainin-2 (F12W mutant) increased its activity against Gram-negative bacteria; however, this increased its selectivity toward erythrocytes, causing significant haemolysis (Matsuzaki et al., 1997). This could be attributed to the bulkiness of Trp compared to Phe and the presence of NH-group in Trp that is capable of forming hydrogen bonds with zwitterionic phospholipids (Creamer and Rose, 1992, Manikandan and Ramakumar, 2004). Further modification through reduction of net charge of F12W mutant (Lys→Glu substitution at 10^{th} position) was shown to reduce the haemolytic effect. However, this made the mutant magainin-2 less effective against Gram-negative bacteria (Matsuzaki et al., 1997). Extensive SAR studies from Zasloff's laboratory led to the development of MSI-78 (also known as pexiganan), a derivative of magainin-2, which showed improved safety/efficacy profile

compared to parent magainin-2 sequence (Gottler and Ramamoorthy, 2009, Ge et al., 1999). However, it failed in the phase III clinical trial for the treatment of infective diabetic foot ulcers.

Typically, natural HDPs display a net positive charge between +2 to +13. It has been widely demonstrated that modification of total net charge of synthetic HDPs through cationic residue substitution enhances electrostatic interaction between HDPs and LPS (Haney et al., 2017, Matsuzaki et al., 1997). However, this approach has shown to increase the toxicity of certain HDPs. For example, magainin-2 analogues with positive charge above +5 were shown to display haemolytic effects (rank order +6 > +5 > +4 > +3) (Dathe et al., 2001). To overcome the inherent issues associated with peptide optimisation, Mishra and Wang (2012) adopted an *ab initio* design approach which involved utilisation of novel database-filtering technology (DFT). This led to the development of a 13-AA long, leucine-rich, anti-MRSA peptide template - termed 'DFTamP1' (Mishra and Wang, 2012). A subsequent study demonstrated that DFT503, an optimised variant of DFTamP1, was shown to be safe and effective in *in vivo* killing of MRSA in a neutropenic mouse model. This anti-MRSA activity was attributed to its eight Leu residues and a single Lys at position 11 (net charge +1) (Mishra et al., 2019). These studies suggested that lower cationic charge and high hydrophobicity is preferred for anti-MRSA synthetic peptides. This strategy could form the basis for the development of species-specific peptide-based therapy against MDR pathogens.

LL-37 is a lone member of the cathelicidin family of HDPs reported in humans (Scott et al., 2002, Harder et al., 1997, Cowland et al., 1995). It was widely studied due to its multi-functional abilities, including microbicidal (Zanetti et al., 1995), anti-cancer, immunomodulatory (Bowdish et al., 2005), chemotactic (De et al., 2000), and wound-healing properties (Ramos et al., 2011). Numerous groups have exploited the

structure of LL-37 to design a range of synthetic antimicrobial analogues through residue substitution (Wang et al., 2019b, Wang et al., 2014). FK-13 (residues 17-29 of LL-37) was identified as a core antimicrobial and anti-cancer domain using nuclear magnetic resonance technique (Wang, 2008a). Subsequently, the deletion of Phe at 17th position led to the development of KR-12, which showed potent antimicrobial efficacy equivalent to LL-37 and FK-13 against *Escherichia coli*, but devoid of toxic activity against host cells (Wang, 2008b). KR-12 and KE-18 analogues were recently shown to possess anti-*Candida* and anti-*Staphylococcal* properties (Luo et al., 2017). Specifically, KE-18 showed anti-biofilm activity even at sub-killing concentration against yeast and bacteria (Luo et al., 2017). Further variants of KR-12 were also reported and the less cationic analogues, a5 and a6, were shown to possess potent immunomodulatory, antibiofilm, antimicrobial, and osteogenic properties (Jacob et al., 2013, Kim et al., 2017, Li et al., 2019a, Fu et al., 2020). Variants of LL-23, corresponding to 23 N-terminal residues of LL-37, were generated through substitution of SeràAla and SeràVal at the 9th position. LL-23V9 peptide was shown to display increased antimicrobial and immunosuppressive activities compared to LL-23 and parent LL-37 (Wang et al., 2012a). Wang's group (Mishra and Wang, 2017, Wang et al., 2019b) have recently demonstrated that titanium surface immobilised FK-16 (a short variant of LL-37) is highly antimicrobial against ESKAPE pathogens. Mohammed et al. (2019) recently demonstrated that FK-16 could be used for repurposing conventional antibiotics such as vancomycin as a strategy to counter AMR. Further improvement of FK-16 by Wang's group (Narayana et al., 2019a) have also led to the development of GF-17, 17BIPHE2, and other related variants of superior efficacy and safety compared to LL-37. Nell et al. (2006) designed a range of short peptides through residue substitution based on the LL-37 sequence for neutralisation of lipopolysaccharide (LPS) and lipoteichoic acid (LTA). P60.4, a 24-AA derivative, was shown to possess similar LPS/LTA neutralisation ability and antimicrobial effects compared to LL-37, but with negligible *in vivo* toxicity toward

audible canal, skin, and eyes. This peptide was subsequently termed as OP-145 and was proven to be safe and efficacious in the treatment of chronic otitis media in phase I/II clinical trials (Malanovic et al., 2015). However, the activity of OP-145 was recently shown to be reduced in human plasma (Riool et al., 2017). Subsequent modification led to the development of synthetic antimicrobial and antibiofilm peptides (SAAPs) such as SAAP-145, -148, and -276, which showed potent anti-biofilm activity against a range of MDR pathogens (Riool et al., 2017).

1.3.3.2. Residue substitution with unnatural amino acids

HDPs are essentially a group of small bioactive molecules made of different combinations of 20 naturally occurring amino acids. The nearly infinite chemical space (20^n) and varying physicochemical properties account for the vast structural and functional diversities of naturally occurring HDPs (Zhao et al., 2013, Wang et al., 2016). However, susceptibility to host cell interaction (e.g. human erythrocytes, albumin, etc.) (Starr et al., 2016, Svenson et al., 2007) and proteolytic degradation from the host and bacterial proteases (e.g. human proteases in serum, staphylococcus aureolysin, pseudomonas elastase, etc.) (Starr and Wimley, 2017, Sieprawska-Lupa et al., 2004, Moncla et al., 2011, Bottger et al., 2017) remains one of the key impediments in translating HDP-based treatment to clinical therapeutics. For instance, the anti-staphylococcal activity of cathelicidin (LL-37) – one of the most widely studied human HDPs – was shown to be inhibited by the proteases produced by *S. aureus*, namely the aureolysin (a metalloproteinase) and V8 protease (glutamylendopeptidase), via cleavage and hydrolysis of the intramolecular peptide bonds (Sieprawska-Lupa et al., 2004).

To overcome this barrier, incorporation of unnatural or non-proteinogenic amino acids has been employed to increase the proteolytic stability and/or antimicrobial efficacy of HDPs. It is known that the antimicrobial efficacy of HDPs is greatly influenced by

the cationicity (Nguyen et al., 2011). To preserve the cationicity and thence efficacy, researchers have attempted to optimise the HDPs by replacing the cationic residues (e.g. lysine) with its analogues such as ornithine, 2,4-diamino-butyric acid (DAB), and 2,3-diamino-propionic acid (DAP), which have three, two, and one methylene (CH_2) groups in the side chain, respectively (Clemens et al., 2017, Arias et al., 2018). Using Trp-rich peptides as the design template, Arias et al. (2018) reported a 4-fold length-dependent increase in the antibacterial activity against *E. coli* when the side chain of lysine was shortened from 4-carbon (lysine) to 1-carbon (DAP). Such effect was likely attributed to an increase in membrane permeabilisation based on calcein leakage study. In addition, a substantial improvement in the stability against trypsin was observed when the side chain of arginine or lysine was shortened (Arias et al., 2018). Oliva et al. (2018) investigated the potential role of integrating unnatural amino acids within the 9-residue synthetic HDPs and discovered that unnatural amino acids such as 2-naphthyl-L-alanine (an aromatic residue) and S-tert-butylthio-L-cysteine residues enhanced the antimicrobial efficacy and proteolytic stability in 10% serum for 1 hour and 16 hours (to a lesser extent). In addition, incorporation of unnatural amino acids dipeptides (tetrahydroisoquionolinecarboxylic acid - octahydroindolecarboxylic acid; or Tic-Oic) within magainin analogues has been shown to induce an amphipathic and loose alpha-helical structure with enhanced antimicrobial potency and selectivity against Gram-positive, Gram-negative and mycobacterium (Hicks et al., 2007).

Unnatural amino acids have been successfully utilised for improving the efficacy and stability of various peptidomimetics (Qvit et al., 2017). For example, Saralasin, a partial angiotensin II receptor agonist, was developed by incorporation of sarcosine (an unnatural amino acid) at a key position in angiotensin II molecule (Gavras and Brunner, 2001). This provided resistance against aminopeptidases and improved bioactivity. Carbetocin, a cyclic 8-AA derivative of oxytocin is currently used for the

treatment of post-partum hemorrhage, was developed through incorporation of unnatural AAs such as methyltyrosine which improved its metabolic stability and overall therapeutic benefits (Gruber et al., 2012).

1.3.3.3. Hybridisation

Hybridisation is a concept used to describe rational combinations of two different peptide sequences of interest, with an aim to improve the therapeutic potential of the hybridised molecules. It is widely known that a cocktail of HDPs are produced at the tissue sites in response to infection (Grassi et al., 2017, Yan and Hancock, 2001). This natural synergism between HDPs was shown to be beneficial to the host, providing the first line of defence against pathogens. This was very well exploited through *in vitro* and *in vivo* studies, which proved that the combination of two HDPs produces strong activity against bacteria (Grassi et al., 2017, Yan and Hancock, 2001). However, this was not deemed as a cost-effective approach and the issue of host toxicity remains unresolved. Hybridisation strategy was shown to circumvent these known issues, which involves the combination of key residues from two to three HDPs of different mechanisms of actions into a single sequence (Almaaytah et al., 2018, Wade et al., 2019, Wang et al., 2019a). Boman et al. (1989) elegantly showed that a hybrid of cecropin-A (1-13) and melittin (1-13) was highly bactericidal and less toxic toward host cells compared to parent cecropin-A and melittin. Subsequent modifications led to the development of numerous short hybrids of cecropin-A and melittin (15 to 18 residues in length), which showed similar activity as the first-generation hybrids (Andreu et al., 1992, Wade et al., 1992). Chimeras of cecropin-A (CA) and magainin-2 (MA) were also developed that exhibited potent antibacterial and antitumor activities. Insertion of hydrophobic residues through residue substitution in the hinge region (at 16^{th} position) of CA(1-8)-MA(1-12) hybrid was shown to improve its antibacterial and antitumor activity with no haemolytic effects (Shin et al., 1999). A recent study has demonstrated that substitution of key residues

in CA(1-8)-MA(1-12), specifically Phe5Lys, Lys7His, Phe13His, Leu14Phe, and His17Leu, could stabilise the alpha-helical conformation, resulting in improved LPS binding affinity, increased bactericidal activity against clinical Gram-negative isolates, and low cytotoxicity (Lee et al., 2016b). Hybrids of human-derived and animal-derived HDPs were also developed to comprise the membrane-lytic and immunomodulatory properties of cationic HDPs. For example, hybrids of cecropin-A (1-8)-LL37(17-20) (Wei et al., 2016), melittin(1-13)-LL37(17-30) (Wu et al., 2014), and BMAP27(9-20)-LL37(17-29) (Tall et al., 2020) were shown to be highly bactericidal and improved the efficacy of conventional antibiotics against a variety of bacteria. Another study involving 'triple hybrid' of cecropin-A, melittin, and LL-37 showed that this approach could significantly enhance the bactericidal against a range of Gram-negative and Gram-positive organisms (Fox et al., 2012). Similarly, Dutta et al. (2016) reported good *in vitro* and *in vivo* efficacies of an antimicrobial CL coated with melimine (derived from melittin and protamine) for treating IK in a rabbit model. These studies have clearly indicated that an optimised rational design approach could enable development of chimeras with improved biological selectivity.

In addition to overcoming the issue of host toxicity (as described above), the hybrid strategy has also led to the development of numerous species-specific and targeted bactericidal peptides to prevent damage to useful microbiome (Kim et al., 2020, Xu et al., 2020, Eckert et al., 2006). Kim et al. (2020) have developed a targeted chimeric peptide for the treatment of *P. aeruginosa* infection. Through phage-display library screening, they identified an outer-membrane porin F (OprF) binding peptide motif, termed PA2. Hybridisation of this tag sequence to a membrane-lytic short peptide, GNU-7, was shown to improve the antimicrobial efficacy of parent GNU-7 by 16-fold toward *P. aeruginosa* both in *in vitro* and *in vivo* model systems. LPS-targeting GNU-7 variants were also developed through hybridisation with lactoferrin (28-34), BPI (84-99), and *de novo* sequence (Kim et al., 2016). Chimeric bactericidal peptides targeted

to *E. faecalis* (Xu et al., 2020) and *S. mutans* (Eckert et al., 2006) were also developed based on the species-specific pheromones. This approach was proven to be highly targeted and would prevent the damage to commensal microbes.

1.3.3.4. L-to-D heterochiral isomerisation

Depending on the geometric arrangement, all naturally occurring amino acids (except for glycine) can exist as stereoisomers, either in L- or D-form, albeit only the L-configuration can be utilised by cells (Aliashkevich et al., 2018). That said, there is emerging evidence showing that most organisms are able to produce D-amino acids, primarily through spontaneous racemisation of L-amino acids or post-translational enzymatic modification (Li et al., 2016). In addition, D-amino acids such as D-alanine and D-glutamic acid are found in peptidoglycan, which is a key component of the cell wall of Gram-positive bacteria. These D-amino acids have been shown to increase resistance to host proteases that usually cleave the peptide bonds between L-amino acids, thereby maintaining their virulence (Aliashkevich et al., 2018).

Capitalising on the evolutionarily advantageous strategy equipped by microbes, L-to-D isomerisation has been utilised to enhance the proteolytic stability of HDPs against a range of host and microbes' proteases (Wade et al., 1990, Cardoso et al., 2018, Carmona et al., 2013, Jia et al., 2017, Manabe and Kawasaki, 2017, Mangoni et al., 2006). L-to-D isomerisation can be utilised to either modify specifically one or several L-amino acids (Jia et al., 2017, Heidary et al., 2018), or the entire sequence of a L-form HDP (Carmona et al., 2013, Jia et al., 2017, Manabe and Kawasaki, 2017). Carmona et al. (2013) demonstrated that L-to-D isomerisation of Panidin-2 (D-Pin2) improved the cell selectivity (i.e. reduced haemolysis) and proteolytic stability in human serum, elastase, and trypsin, while maintaining the antimicrobial activity against a range of Gram-positive and Gram-negative bacteria. In a similar vein, Jia et al. (2017) reported an improved stability of D-amino acid derivative of polybia-CP

(which was originally derived from the venom of social wasp *Polybia paulista*), in chymotrypsin and trypsin for 1 hour and 6 hours and reduced haemolytic activity (D-lysine derivative). In addition to the beneficial effect of proteolytic stability and/or cell selectivity, the D-form amino acids may enhance the antimicrobial efficacy of HDPs. For example, the D-form KLKLLLLLKLK-NH2 (derived from sapesin B) was shown to exhibit increased antimicrobial efficacy against *S. aureus* (due to increased interaction with the peptidoglycan), *E. coli* and *Candida albicans* when compared to the L-form (Manabe and Kawasaki, 2017). However, the enhanced antimicrobial efficacy was not observed in other tested D-forms of HDPs such as mastoparan M and temporin A, suggesting that the D-isomerisation effect is sequence-dependent (Manabe and Kawasaki, 2017).

L-to-D isomerisation has also been shown to confer unique changes to the peptide-folding and secondary structure of HDPs (Mangoni et al., 2006, Jia et al., 2017, Wade et al., 1990). Based on circular dichroism analysis, the D-form derivatives of naturally occurring alpha-helical HDPs typically exhibit a left-hand alpha-helical spectrum (instead of a right-hand spectrum) whereas partial D-isomerisation of HDPs may result in some degree of loss of alpha-helicity, depending on the position and number of D-amino acids being introduced (Wade et al., 1990, Jia et al., 2017, Mangoni et al., 2006). Such changes in the secondary structure are likely accountable for the reduced host toxicity and improved cell selectivity in some HDPs (Jia et al., 2017).

This strategy was found to be successful in the development of daptomycin, an antibacterial cyclic lipopeptide, which was approved by the US Food and Drug Administration (FDA) in 2003 for the treatment of skin and systemic Gram-positive infections (Liu et al., 2011, Qvit et al., 2017). Structurally, daptomycin is comprised of 13 residues including D-alanine and D-serine (Heidary et al., 2018). In addition, it also

contains non-canonical amino acids such as ornithine, L-kynurenine, and L-3-methylglutamic acid (Heidary et al., 2018).

1.3.3.5. C- and N-terminal modification

A range of N- and C-terminal modification strategies have been proposed to enhance the antimicrobial efficacy and/or cell selectivity of natural and synthetic HDPs (Oliva et al., 2018, Dahiya et al., 2018, Wang, 2012). Amongst all, N-terminal acetylation (CH_3CO-) and C-terminal amidation (-NH_2) are the two most commonly attempted strategies (Oliva et al., 2018). N-acetylation is a common protein modification observed in eukaryotic and prokaryotic cells (Ree et al., 2018). By neutralising the positive charge (NH_3^+) at the N-terminal, N-acetylation can result in a range of irreversible changes to the protein properties, including the folding, stability, and protein-protein interactions (Ree et al., 2018). Saikia et al. (2017) examined the antimicrobial efficacy and salt sensitivity of *E. coli*-derived MreB (a bacterial cytoskeleton protein found in non-spherical cells) and its N-acetylated analogues and found that N-acetylated W-$MreB_{1-9}$ demonstrated a higher antimicrobial efficacy (in salt) compared to W-$MreB_{1-9}$. However, N-acetylation may result in a decrease in antimicrobial efficacy of certain synthetic HDPs due to a reduction in the overall cationicity (Saikia et al., 2017, Crusca et al., 2011), suggesting that the benefit of this modification strategy is only selective for certain HDPs.

Table 1.5. Strategies in translating the therapeutic potentials of HDPs.

Authors	Year	HDPs template	Strategies	Biological Effects
Residue substitution				
Lee et al.	2013	HP ribosomal protein 1	Pro substitution	Increased efficacy
Wang et al.	2012	LL-37	Ala/Val substitution	Increased efficacy
Blondelle et al.	1993	Melittin	Trp substitution	Reduced toxicity
Hybridisation				
Wei et al.	2016	Cecropin and LL-37	Hybridisation	Increased efficacy and reduced toxicity
Wu et al.	2014	Melittin and LL-37	Hybridisation	Increased efficacy and reduced toxicity
Boman et al.	1989	Cecropin and melittin	Hybridisation	Improved efficacy and reduced toxicity
Unnatural AA				
Arias et al.	2018	Indolicidin	Ornithine, DAB, DAP, Agb and hArg	Improved efficacy against GN and proteolytic stability
Clemens et al.	2017	Cecropin and magainin	Ornithine	Antimicrobial and anti-biofilm efficacies
Hicks et al.	2007	Magainin	Tic-Oic	Increased efficacy and reduced toxicity
L-to-D isomerisation				
Jia et al.	2017	Polybia-CP	LDI	Improved proteolytic stability and reduced toxicity
Manabe et al.	2017	Sapesin B	LDI	Improved efficacy against GP, GN and fungi
Carmona et al.	2013	Pandinin 2	LDI	Reduced host tissue toxicity
C- and N- terminal modifications				
Saikia et al.	2017	MreB	N-acetylation	Improved antimicrobial efficacy in salt
Falciani et al.	2014	M33	C-pegylation	Increased proteolytic stability
Dennison & Phoenix	2011	Modelin-5	C-amidation	Improved stabilisation of alpha-helix and antimicrobial efficacy

Cyclisation				
Mwangi et al.	2019	Cathelicidin-BF	Cyclisation	Antimicrobial and antibiofilm efficacies against MDR-GN and good stability
Scudiero et al.	2015	HBD-1 and -3	Cyclisation	Increased proteolytic stability
Fernandez-Lopez et al.	2001	De novo	Cyclisation of D,L-alpha peptides	Increased efficacy
Incorporation with nanoparticles				
Comune et al.	2017	LL-37	Gold NP	Improved wound healing
Casciaro et al.	2017	Esculentin-1a	Gold NP	Improved antimicrobial efficacy, wound healing and stability
Chereddy et al.	2014	LL-37	PLGA NP	Improved wound healing
Smart design with artificial intelligence technology				
Yount et al.	2019	5200 12-mer peptide sequence	SVM-based classifier	Identification of a unifying alpha-core signature with good correlation with ability to generate NGC
Lee et al.	2016	572 alpha-helical peptides	SVM-based classifier	Accurate prediction of peptide ability to generate NGC
Cherkasov et al.	2009	Random 9-mer peptide database	QSAR model using ANN	Generation of active synthetic peptides against MDR GP and GN, with low toxicity

HP = *Helicobacter pylori*; GP = Gram-positive bacteria; GN = Gram-negative bacteria; DAB = 2,4-diamino-butyric acid; DAP = 2,3-diamino-propionic acid (DAP); Agb = (S)-2-amino-4-guanidinobutyric acid; hArg = homo-arginine; Tic-Oic = tetrahydroisoquionolinecarboxylic acid-octahydroindolecarboxylic acid dipeptide; HBD = Human-beta-defensin; PLGA = Poly lactic-co-glycolic acid; SVM = support vector machine; NGC = Negative Gaussian curvature; ANN = Artificial neural network; MDR = Multidrug resistant

On the other hand, C-amidation is a common post-translational modification that is widely observed in nature, including the natural synthesis of HDPs (Wang, 2012). C-amidation has been shown to improve the antimicrobial efficacy of certain HDPs, including aurein (Mura et al., 2016), melittin (Irudayam and Berkowitz, 2012), modelin-5 (Dennison and Phoenix, 2011), anoplin (Dos Santos Cabrera et al., 2008), and esculentin-1 (Islas-Rodriguez et al., 2009), amongst others. The enhanced antimicrobial efficacy of these HDPs is likely ascribed to the increased alpha-helix stability at the peptide-membrane interfaces, enabling a greater membrane disruption and pore formation (Dos Santos Cabrera et al., 2008, Dennison and Phoenix, 2011, Mura et al., 2016, Irudayam and Berkowitz, 2012). In addition, Oliva et al. (2018) demonstrated that simultaneous N-acetylation and C-amidation enhanced the proteolytic stability of HDP derived from human apolipoprotein B by more than four-fold when exposed to fetal bovine serum 10% for 1 hour. Similarly, the proteolytic stability of tachyplesin I (a beta-hairpin HDP from the horseshoe crab, *Tachypleus tridentatus*) in fresh human serum was significantly enhanced using the similar N-acetylation and C-amidation strategy (Kuzmin et al., 2017).

Other N- or C-terminal modification strategies have also been described in the literature, including N-methylation of certain cyclic HDPs to enhance the antimicrobial efficacy (Dahiya et al., 2018), introduction of 6-aminocaproic acid at the N- and C-terminals to protect HDPs from the action of exopeptidases (Oliva et al., 2018, Purwin et al., 2017), and pegylation of the C-terminus of M33, a branched peptide, to increase the resistance against *P. aeruginosa* elastase (Falciani et al., 2014).

1.3.3.6. Cyclisation

Cyclisation is a common phenomenon observed in natural HDPs that can exist in three main forms: (a) sidechain to sidechain; (b) backbone to backbone; and (c) sidechain to backbone (Wang, 2012). It has been shown to demonstrate several

favorable biological properties, including enhanced antimicrobial efficacy, stability against proteases (due to conformational rigidity), enhanced cell selectivity, and reduced host toxicity (Zorzi et al., 2017, Wang, 2012), rendering it an attractive strategy for translating HDPs from bench to bedside. Some of the notable examples of cyclic glyco- or lipopeptides that are already in clinical use include vancomycin, daptomycin, and colistin/polymyxin, which are commonly used as last resorts for combatting MDR bacteria, albeit their widespread use is hindered by the inherent toxicity and emergence of AMR (Zorzi et al., 2017, Falagas and Kasiakou, 2005, Johnson et al., 1990).

In view of the structural stability, Dathe et al. (2004) were able to create a series of short cyclic hexapeptides (based on AcRRWWRF-NH_2) with enhanced antimicrobial efficacy (up to >16-fold increase) against *Bacillus subtilis* and *E. coli* compared to the linear form, though the haemolytic activity was increased by threefold (Dathe et al., 2004). It was also found that the antimicrobial activities of those small Arg/Trp-rich cyclic peptides were influenced by the self-assembling behavior of peptides at the bacterial membrane instead of their hydrophobic surface area, amphiphilicity, and ring size (Bagheri et al., 2018). In addition, a number of small cyclic D,L-alpha-peptides (with six or eight alternating D- and L-form residues) have also demonstrated strong antimicrobial efficacy against Gram-positive and/or Gram-negative bacteria via self-assembly on the bacterial membranes as organic nanotubules, which could increase membrane permeability and disrupt transmembrane ion potentials with resultant cell lysis (Fernandez-Lopez et al., 2001, Dartois et al., 2005). Furthermore, molecular dynamic simulations and biophysical assays have provided further supportive evidence that cyclic peptides are able to bind to negatively charged membrane more strongly than the linear peptides and adopt a beta-sheet structure at the membrane surface (Mika et al., 2011).

Another form of cyclisation that is found abundantly in natural HDPs, mainly in defensins, is the disulfide intramolecular cross-link between cysteine residues, which has been shown to enhance proteolytic stability (Falanga et al., 2017, Menendez and Brett Finlay, 2007, Sher Khan et al., 2019, Scudiero et al., 2015). Inspired by the nature, Scudiero et al. (2015) engineered a 17-residue cyclic synthetic hybrid HDP, based on the internal hydrophobic domain of human-beta defensin (HBD)-1 and positively charged C-terminal of HBD-3 (RRKK residues), and demonstrated good antimicrobial efficacy against Gram-positive and Gram-negative bacteria and herpes simplex virus, with low toxicity and good proteolytic stability. Similarly, Mwangi et al. (2019) successfully developed a cyclic HDP-based molecule called ZY4 by introducing a disulfide bond to a derivative of cathelicidin-BF, which is an antimicrobial peptide derived from the snake venom of *Bungarus fasciatus*. This molecule was shown to exhibit significant *in vivo* antimicrobial efficacy against MDR *P. aeruginosa* and *A. baumannii* with high stability in mice lung infection and septicemia models (Mwangi et al., 2019).

1.3.3.7. Incorporation with nanoparticles (NPs)

Nanotechnology is a rapidly growing field in biotechnology that involves characterisation, manipulation and synthesis of materials that are in nanoscales (or one billionth of meter; 10^{-9} m) (Bayda et al., 2019). NPs, with sizes ranging from 1 nm to 100 nm, can exist in many forms, including lipid-based, metal-based, carbon-based, ceramics, semiconductor, and polymeric NPs (Khan et al., 2019). It has increasingly been applied in the field of antimicrobials, either employed as antimicrobial agents or nano-carriers for drug/peptide delivery in view of their enhanced protection against extracellular degradation, improved bioavailability and cell selectivity (Reshma et al., 2017, Natan and Banin, 2017, Biswaro et al., 2018, Lakshminarayanan et al., 2018). Recently, Biswaro et al. (2018) have provided an excellent review on the role of nanotechnology in delivering HDPs. To avoid any

significant overlap, this section aims to only recapitulate the fundamental principles of NPs and provide some notable examples regarding the potential values of incorporating NPs with HDPs.

In principle, there are two types of nano-delivery systems: (a) passive delivery where the intended drugs/peptides are encapsulated within the nanocarriers through hydrophobic interaction without any surface modification; and (b) active delivery where the drugs/peptides are directly conjugated with the nanocarriers with surface modification with ligands or other moieties to facilitate delivery to the targeted site (Patra et al., 2018). Among all, LL-37 and its mimics are some of the most commonly explored HDPs that have been incorporated with different types of NPs, including polymeric NPs [e.g. poly lactic-co-glycolic acid (PLGA)] (Chereddy et al., 2014), gold NP (Ferreira et al., 2018a, Comune et al., 2017), and magnetic NPs (Wnorowska et al., 2020).

PLGA is a FDA-approved biodegradable and biocompatible polymer that has demonstrated promising potential as a drug delivery carrier (Makadia and Siegel, 2011). Chereddy et al. (2014) described using PLGA as a nanoparticle carrier for delivering a sustained release of LL-37 treatment. Compared to PLGA or LL-37 alone, PLGA-LL37 nanoparticles were reported to expedite the wound healing process with significantly higher collagen deposition, re-epithelialisation and neovascularisation (Chereddy et al., 2014). It also demonstrated better antimicrobial activity against *E. coli* compared to PGLA alone, though the efficacy was lower than LL-37 alone (Chereddy et al., 2014). Cruz et al. (2017) similarly reported that the encapsulated form of GIBIM-P5S9K peptide within PLGA or polylactic acid exhibited around 20 times stronger antimicrobial efficacy against methicillin-resistant *S. aureus* (MRSA), *P.* aeruginosa, and *E. coli* when compared to the free peptide.

In addition, gold NPs have been increasingly applied in the field of HDPs (Yeh et al., 2012). Comune et al. (2017) demonstrated that LL-37 conjugated with gold NPs (via an additional cysteine residue at the C-terminus of LL-37) demonstrated superior *in vitro* and *in vivo* wound healing properties compared to LL-37 alone. This was attributed to the prolonged phosphorylation of epidermal growth factor receptor (EGFR) and extracellular-signal-regulated kinase (ERK)1/2, which increased the migration of keratinocytes. Gold NPs have also been used as nanocarriers for other HDPs such as Esc(1-21), a derivative of a frog skin HDP called esculentin-1a (Casciaro et al., 2017). Compared to Esc(1-21), it was found that the conjugated form of Esc(1-21) with gold NPs via a poly-ethylene glycol linker improved the antimicrobial efficacy against *P. aeruginosa* by around 15-fold, with increased resistance to proteolytic degradation (Casciaro et al., 2017). Certain peptides have also demonstrated self-assembling ability as a nanocarrier for drug delivery (Biswaro et al., 2018).

1.3.3.8. Smart design using artificial intelligence (AI) technology

AI serves as one of the major breakthroughs in the mankind's history. Long been deployed in the automobile and technology industries, AI has only started gaining traction in the field of science and medicine, owing to the advancement in computer power, availability of big data, publicly available neural networks, and improvement in AI algorithms using machine learning and deep learning (Hamet and Tremblay, 2017, Ting et al., 2019a, Schneider et al., 2019, LeCun et al., 2015, Ting et al., 2020a, Ting et al., 2021c). In view of the infinite chemical space and complex SAR of natural and synthetic HDPs, AI serves as an attractive solution to identify and predict novel peptide sequences with potentially good antimicrobial efficacy (Cardoso et al., 2019b, Cherkasov et al., 2009, Lee et al., 2016a, Yount et al., 2019).

To date, a number of machine learning algorithms such as artificial neural network (ANN) (Cherkasov et al., 2009, Lata et al., 2007), support vector machine (SVM)-based classifier (Lee et al., 2016a, Yount et al., 2019, Lata et al., 2007), quantitative matrices (Lata et al., 2007), and fuzzy K-nearest neighbor (Xiao et al., 2013) have been developed to search for the ideal synthetic HDPs. Cherkasov et al. (2009) trained an atomic-based QSAR model using ANN and inductive chemical descriptors based on two large 9-mer peptide libraries. The model was then tested against 200 peptides that were chosen from a virtual library of 100,000 random 9-mer peptides. The model not only successfully predicted the antimicrobial efficacy of the synthetic peptides but also identified potent peptide candidates (HHC-10 and HHC-36) which were highly active against a range of Gram-positive and Gram-negative superbugs, with low risk of toxicity (Cherkasov et al., 2009).

On the other hand, Lee et al. (2016a) developed a SVM-based classifier coupled with Pareto-optimisation (Shoval et al., 2012) to deduce the functional and structural similarities of alpha-helical HDPs. By employing antimicrobial assays and small-angle X-ray scattering, it was found that the SVM distance to hyperplane σ correlated strongly with the ability of HDP in generating a negative Gaussian curvature (NGC), which is commonly responsible for the membrane disruption mechanism of HDP (Lee et al., 2016a). Subsequently, Yount et al. (2019) were able to identify a unifying physicochemical characteristic of alpha-helical HDPs in a 3-dimensional space, termed the alpha-core signature, using knowledge-based annotation and pattern recognition analysis of bioinformatics databases. The antimicrobial efficacy of this alpha-core signature (i.e. the ability to induce NGC) was further validated with the previously developed SVM-based classifier (Yount et al., 2019).

1.3.4. Therapeutic potentials of HDPs for ocular surface diseases

A wide range of HDPs have so far been developed but none has reached the market. In this section, some of the key HDP-based molecules that have completed *in vivo* animal studies and are in the developmental pipeline for treating OS diseases are highlighted. These include B2088 branched peptide, Esculentin1-21(NH2), RP444, melimine/Mel4 antimicrobial coating for CL, epsilon-lysylated melittin (MEL-4), and endogenous LL-37.

1.3.4.1. B2088 branched peptide

B2088 is a covalent dimeric peptide that is derived from the C-terminal of HBD-3 [peptide sequence: $(RGRKVVRR)_2KK$] (Zhou et al., 2011). The development of this branched peptide was started in 2007 where Liu et al. (2008) demonstrated that the linear form of HBD3 maintained similar antimicrobial efficacy and exhibited lower cytotoxicity and haemolytic activity compared to the native form of HBD3, after refining the hydrophobicity and substituting the cysteine residues with various amino acids. Such properties were postulated to be related to the removal of the disulfide bridges and the loss of secondary structure. Bai et al. (2009) further enhanced the antimicrobial activity and reduced the host toxicity of linear HBD3 analogues by shortening the HBD3 to 10 amino acids from the C-terminal end. Taking it further, the antimicrobial efficacy of the truncated HBD3 were further optimised via dimerisation at the lysine, which yielded the final lead compound of B2088 (Zhou et al., 2011, Lakshminarayanan et al., 2016).

B2088 has been shown to demonstrate strong antimicrobial activity against Gram-negative bacteria, particularly *P. aeruginosa* (Zhou et al., 2011, Lakshminarayanan et al., 2016). It exerts its bacterial killing through the binding of lipid A and disruption of supramolecular organisation of lipopolysaccharides, a major component of the

outer membrane of Gram-negative bacteria. In addition, B2088 strong synergism with various antibiotics through time-kill and checkerboard assays. This was further validated in an *in vivo* murine *P. aeruginosa* keratitis study where B2088 0.05%-gatifloxacin 0.15% combination treatment reduced the bacterial burden of corneal infection by an additional 1 LogCFU compared to gatifloxacin 0.3% alone (Lakshminarayanan et al., 2016).

1.3.4.2. Esculentin-1a(1-21)NH2

The skin of amphibians contains a rich source of HDPs (Ladram and Nicolas, 2016). Esculentin-1a is a type of frog-derived HDP isolated from the skin of *Rana esculenta*, or now known as *Pelophylax lessonae/ridibundus*. The modified version, Esculentin-1a(1-21)NH_2, is composed of the first 20 amino acids of esculentin-1a with a glycinamide residue at the C-terminal end (peptide sequence: GIFSKLAGKKIKNLLISGLKG-NH_2*)* (Kolar et al., 2015). It has been shown to demonstrate strong *in vitro* antimicrobial activity against various *P. aeruginosa* laboratory strains (both invasive and cytotoxic strains) and clinical strains (isolated from eyes with keratitis and conjunctivitis)*,* and *Staphylococci species* (with a MIC range of 1 – 16 μM) (Kolar et al., 2015). In an *in vivo* murine bacterial IK model infected with cytotoxic *P. aeruginosa* strain, topical treatment of esculentin-1a(1-21)NH_2 significantly reduces the bacterial load, clinical severity and recruitment of inflammatory cells to the infected corneas measured by the relative myeloperoxidase activity (Kolar et al., 2015). In addition, it was shown to exhibit anti-biofilm activity against *P. aeruginosa* and prolong the survival of PAO1-infected mice in both sepsis and pneumonia models (Luca et al., 2013). The potent activity against both planktonic and sessile forms of *P. aeruginosa* was ascribed to its underlying membrane perturbation activity (Luca et al., 2013).

1.3.4.3. RP444

The development of RP444 was inspired by the “freedom from infection” observed in Cecropia moth and African clawed frog, which is attributed to the cecropins and magainins peptides, respectively (Zasloff, 2019). RP444 is a 23-amino acid designed HDP primarily composed of phenylalanine, alanine and ornithine, which is an unnatural amino acid used to replace lysine residue to enhance antimicrobial activity and proteolytic stability (peptide sequence: FAOOFAOOFOOFAOOFAOFAFAF) (Clemens et al., 2017). This designed HDP possesses a broad-spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacteria (MIC ranges between 4 – 64 μg/ml) and anti-biofilm efficacy. Similar to other natural and synthetic HDPs, RP444 exhibits rapid bacterial killing within 30-60 mins with no risk of developing resistance. Further *in vivo* murine bacterial keratitis study showed that RP444 was able to significantly reduce the bacterial load and clinical severity of *P. aeruginosa* keratitis and reduce inflammatory cell infiltration towards the infected site (Clemens et al., 2017).

1.3.4.4. Melimine and Mel4 antimicrobial coating for contact lenses

Melimine is a 29-amino acid cationic synthetic HDP derived from melittin (from honeybee venom) and protamine (from salmon sperm) (Willcox et al., 2008). This hybrid HDP combines the C-terminals of both melittin and protamine, yielding a total cationic charge of +14 (peptide sequence: TLISWIKNKRKQRPRVSRRR RRRGGRRRR). When attached to CLs, either through adsorption or covalent binding, melimine demonstrates higher antimicrobial activities against both Gram-positive and Gram-negative bacteria than melittin or protamine alone (Willcox et al., 2008). In addition, the haemolytic activity of melimine is significantly lower than melittin. Furthermore, *in vivo* rabbit models successfully showed that melimine-coated CLs were safe to wear and they prevented bacterial growth on CLs, which consequently reduced the rate and severity of adverse reactions such as CL-induced

acute red eye (CLARE), CL-induced peripheral ulcers (CLPUs) and IK (Cole et al., 2010, Dutta et al., 2014, Dutta et al., 2016). This suggests that hybridisation of two different HDPs serves as a novel strategy to enhance antimicrobial efficacy and reduce toxicity.

However, when the melimine-coated CLs were tested in a human clinical trial, these CLs were paradoxically associated with significantly higher corneal staining compared to uncoated lenses at day 1 (Dutta et al., 2014). To overcome this unforeseen corneal toxicity, the same research group has further refined the hybrid HDP, which has led to the creation of Mel4 – a truncated version of melimine with +14 net charge (peptide sequence: KNKRKRRRRRRGGRRRR) (Dutta et al., 2017). This modified HDP exhibits modest antimicrobial activity against a broad range of Gram-positive and Gram-negative bacteria, with good *in vivo* safety demonstrated in rabbit and human trials (Dutta et al., 2017). The mechanism of action of Mel4 against *P. aeruginosa* was found to be related to the neutralisation of LPS and disruption of cytoplasmic membrane whereas its action against *Staphylococcus aureus* was likely attributed to the release of autolysins with resultant cell death instead of pore formation (Yasir et al., 2019a, b).

1.3.4.5. Epsilon lysylated melittin (MEL-4)

Being as one of the main basic and cationic amino acids, lysine serves as a major constituent of many naturally occurring and synthetic HDPs (Jin et al., 2016, Bradshaw, 2003). In addition to the L- and D-form, lysine can also exist in epsilon form (Ɛ-) where the NH_2 group at the side chain of L-lysine is linked to the alpha-carbon. Ɛ-Poly-L-lysine (EPL) is a basic polyamide consisting of 25-30 Ɛ-lysine that is naturally produced by *Streptomycetaceae* and *Ergot* fungi (Shukla et al., 2012). It is commonly used as a food preservative with strong antimicrobial activity (Geornaras et al., 2007, Shima et al., 1984). Compared to alpha-poly-L-lysine, EPL exhibits

enhanced antimicrobial efficacy against a range of Gram-positive and Gram-negative bacteria (Shima et al., 1984, Venkatesh et al., 2017). Employing the similar strategy, Mayandi et al. (2020) explored the selective incorporation of Ɛ-lysine in melittin, which is a potent yet toxic HDP that is found in honeybee venom. They showed that Ɛ-lysylation of melittin, in particular MEL-4 (different from the Mel4 described above) improved the cell selectivity of the synthetic HDP towards a range of Gram-positive and Gram-negative bacteria with reduced host cytotoxic and haemolytic activities, whilst maintaining the *in vivo* efficacy of melittin (Mayandi et al., 2020). This suggests that Ɛ-lysylation may serve as a novel strategy for improving the cell selectivity in lysine-rich HDPs.

1.4. Aims

In light of the evidence presented above, it is apparent that there is a significant gap in the literature concerning the epidemiology and outcome of IK, particularly in the UK. While adjuvant therapies such as therapeutic corneal cross-linking and amniotic membrane transplantation have been shown to improve the outcome of IK, high-quality evidence remains lacking in the literature. In addition, there is an unmet need for new and novel antimicrobial therapies for IK.

Bearing these issues in mind, the aims of this PhD work are fourfold. The first part of this work aimed to determine the epidemiology, risk factors, clinical outcomes and antimicrobial resistance in relation to IK. The second part aimed to systematically examine the effectiveness and safety of two adjuvant antimicrobial treatment, including therapeutic corneal cross-linking and amniotic membrane transplant for treating IK. The final part of this work aimed to develop a novel HDP-based antimicrobial therapy for treating IK and to decipher the underlying mechanism of

action of the HDP using biophysical assays and molecular dynamics simulation studies.

1.5. Hypotheses

This PhD work aimed to examine the following hypotheses:

1. Infectious keratitis represents a significant ocular morbidity in the UK, and it poses significant negative impact on the patient and the healthcare system.
2. Surgical interventions such as therapeutic corneal cross-linking and amniotic membrane transplant serve as useful adjuvant treatment to expedite the corneal healing in IK.
3. Rational hybridisation of human-derived host defense peptides, particularly human beta-defensins and human cathelicidin (LL-37), can lead to the development of efficacious and safe topical antimicrobial therapy for treating infectious keratitis.
4. Molecular dynamics simulations study can expedite the optimisation of the developed hybrid peptides and help decipher the underlying mechanism of action.

CHAPTER 2

A 12-Year Analysis of Incidence, Microbiological Profiles, and In Vitro Antimicrobial Susceptibility of Infectious Keratitis: The Nottingham Infectious keratitis Study

2.1. Introduction

Infectious keratitis (IK) represents a major cause of corneal blindness globally, accounting for ~3.2% of all blindness (Ting et al., 2021j). It has also been estimated to cause 1.5-2.0 million monocular blindness each year. Based on the limited evidence in the literature, the incidence of IK has been estimated at 0.04-8.0 per 1000 people per year, with a substantially higher rate noted in developing countries such as India, Nepal, and Burma (Ting et al., 2021j).

A wide array of microorganisms, including bacteria, fungi, viruses, and parasites, notably Acanthamoeba, have been implicated in IK. In view of the diverse causative microorganisms and potentially rapid clinical progression, intensive broad-spectrum antimicrobial treatment, either with cephalosporin/aminoglycoside dual therapy or fluoroquinolone monotherapy, is usually commenced to provide an initial comprehensive coverage for IK (Austin et al., 2017a, McDonald et al., 2014). Uncommonly, adjuvant therapies such as tetracyclines (protease inhibitors), amniotic membrane transplantation, and the recently introduced modality of therapeutic corneal cross-linking (PACK-CXL) may be required to halt the progression of IK (Mencucci et al., 2011, Gicquel et al., 2007, Ting et al., 2019e, Robaei et al., 2016).

The diagnosis of IK is primarily made on clinical grounds, supplemented by microbiological investigations such as corneal scraping for microscopy, culture and sensitivity testing (Ung et al., 2019b, Ting et al., 2021f). Depending on the geographical and temporal variations, the profile of causative microorganisms of IK may differ significantly across different regions (Shah et al., 2011). For instance, fungi were shown to be the most common organism for IK in China and India whereas bacteria were most commonly identified in USA and UK (Ung et al., 2019b). In addition, the *in vitro* antimicrobial susceptibility and resistance of ocular isolates similarly varied significantly across the world, with the rate of methicillin-resistant *Staphylococcus aureus* (MRSA) ranging from 0.1% to 36.6% (Ting et al., 2018, Thomas et al., 2019). Moreover, the proportion of multidrug resistant (MDR) ocular isolates is reportedly rising in some regions (Thomas et al., 2019).

To date, there are only two studies in the literature that reported the incidence of IK in the UK, which was estimated at 3.6-52.1 per 100,000 population/year during the period of 1995-2006 (Seal et al., 1999, Ibrahim et al., 2012). A number of studies have recently examined the microbiological profiles and/or *in vitro* antibiotic susceptibility and resistance profiles of IK in the UK (Orlans et al., 2011, Tan et al., 2017, Ting et al., 2018, Tavassoli et al., 2019). Within the region of Nottingham, UK, the most recent review on IK was conducted during the period of 2007-2010 and only focussed on severe and sight-threatening cases (Otri et al., 2013).

The aim of this study was to provide an up-to-date and comprehensive analysis on the incidence, microbiological profiles, and in vitro antimicrobial susceptibility and resistance of IK in Nottingham, UK, over the past 12 years and to compare the findings with the recent literature.

2.2. Materials and methods

2.2.1. Case identification and data collection

This was a retrospective study of all patients who were diagnosed with IK and underwent corneal scraping between July 2007 and October 2019 (a 12-year period) at the Queen's Medical Centre (QMC), Nottingham. Cases were identified through the local microbiology electronic database. QMC was the only tertiary referral centre for managing ophthalmic diseases in Nottingham. The eye casualty embedded within the QMC was open 24/7 to manage patients with emergency ophthalmic conditions, including infectious keratitis. There were 2 other nearby hospitals in the East Midlands regions, including Derby Royal Hospital and Kings Mill Hospital, but they covered a different subset of the population and were not included in Nottingham population or the local IK database.

Based on the departmental guideline for IK, all patients presented with moderate sized corneal ulcers (>1 mm diameter) or atypical presentation of corneal ulcer were subjected to microbiological investigation, which included corneal scraping for microscopy (with Gram staining), microbiological culture and sensitivity testing. Corneal scrapes were inoculated on chocolate agar (for fastidious organisms), blood agar (for bacteria), and Sabouraud dextrose agar (for fungi). For suspected cases of Acanthamoeba keratitis, non-nutrient *Escherichia coli*-enriched agar plate was used for inoculation. All cultures were incubated for at least 1 week (and up to 3 weeks for suspected Acanthamoeba keratitis). The identity of the microorganisms was confirmed through standard culture and bacteriology tests. For example, *S. aureus* was identified by cultural characteristics and positive Pasteurex test whereas *Streptococcus pneumonia* was identified by cultural characteristics and sensitivity to optochin disc. Corneal scraping was repeated in the same eye when the patient was

unresponsive to treatment regardless of positive or negative outcome of the first culture. These cases were only counted as one clinical episode.

Causative microorganisms were categorised into Gram-positive and Gram-negative bacteria, fungi, and Acanthamoeba. Polymicrobial keratitis was defined as IK caused by two or more types of microorganisms simultaneously during the same infective episode. Combined cefuroxime and gentamicin/amikacin were used for deemed sight threatening keratitis (greater than 1 mm lesion, location within the central 6 mm zone and/or related to contact lens wear); or levofloxacin monotherapy for non-sight threatening keratitis (infiltrate size of 1 mm or less, peripheral location and not related to contact lens wear) were the first-line antimicrobial therapy used during the entire study period. *In vitro* antimicrobial susceptibility and resistance were determined using the standard disc diffusion assay or Microscan (MIC) and interpreted according to the clinical breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical_breakpoints/). Multidrug resistance (MDR) was defined as resistance to three or more classes of antibiotic.

2.2.2. Calculation of the population

The population in Nottingham was estimated at between 300,000 and 328,000 people during the study period (https://www.ukpopulation.org/nottingham-population/), and these figures were used to estimate the incidence of IK within the region of Nottingham, UK. For study years of 2007 and 2019 (without the full-year data), the incidence was extrapolated from 6 months' and 10 months' data, respectively. This was because the electronic database was only introduced in July 2007 and the study was concluded in October 2019.

Ethical approval was waived by the local research ethics committee as this retrospective study was classified as a service evaluation (reference number: 19-265C). The study was conducted in accordance with the tenets of Declaration of Helsinki.

2.2.3. Statistical analysis

For descriptive and analytic purposes, the study was divided into two time periods, 2007-2013 (which included the study period of previous study) (Otri et al., 2013) and 2014-2019. Statistical analysis was performed using SPSS version 26.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). Comparison between groups was conducted using Pearson's Chi square or Fisher's Exact test where appropriate for categorical variables and unpaired T test or Mann-Whitney U test for continuous variables. Normality of data distribution was assumed if the skewness and kurtosis z-values were between -1.96 and +1.96 and the Shapiro-Wilk test p-value was >0.05. All continuous data were presented as mean ± standard deviation (SD) and/or 95% confidence interval (CI). Pearson's correlation coefficient (*r*) analysis was performed to examine the incidence of IK over time and was interpreted as follows: weak (*r*=0.00-0.40), moderate (*r*=0.41-0.69), and strong (*r*=0.70-1.00), with negative values being interpreted in the same way (Schober et al., 2018). P-value of <0.05 was considered statistically significant.

2.3. Results

2.3.1. Overall description and incidence of IK

During the 12-year study period, a total of 1400 corneal scrapes were performed in patients with IK; the mean age was 49.9 ± 22.2 years and 50.4% were male. There were 67 cases where repeat corneal scrapings were performed in the same eye. On no occasion were both cultures positive. After excluding 67 repeat corneal scrapings,

there were a total of 1333 cases of IK. The overall incidence of IK in the Nottingham region was estimated at 34.7 per 100,000 population/year (95% CI, 32.4 to 37.1 per 100,000 population/year), with a stable trend observed over time (r = -0.08; p=0.79; **Figure 2.1**).

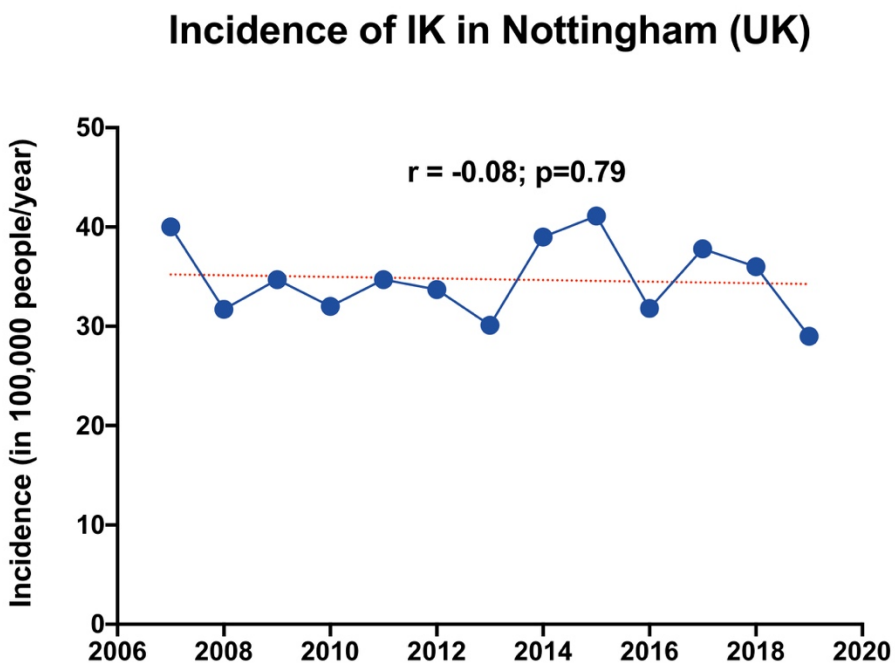


Figure 2.1. Annual incidence of IK in Nottingham in 2007-2019.

2.3.2. Types of causative organisms

Of all 1333 corneal scrapes, 502 (37.7%) were culture-positive and 572 causative microorganisms were identified (**Table 2.1**). Gram-positive bacteria (308, 53.8%) were most commonly isolated, followed by Gram-negative bacteria (223, 39.0%), Acanthamoeba (24, 4.2%), and fungi (17, 3.0%). In terms of specific isolates, *Pseudomonas aeruginosa* (135, 23.6%), *S. aureus* (91, 15.9%), and *Streptococci spp.* (77, 13.5%) were the three most common causative microorganisms identified. Sixty (4.5%) cases were of polymicrobial origin (caused by ≥2 different

microorganisms), with 50 (3.8%) cases having two causative microorganisms and 10 (0.8%) cases having three causative microorganisms. Of the 60 cases, the majority (57, 95%) were mixed bacteria / bacteria infection with only 3 (5%) cases of mixed fungi / bacteria infection. The most common combination of isolates for polymicrobial cases were *Streptococci spp.* combined with coagulase-negative staphylococcus (9, 15%). There was a significant increase in *Moraxella spp.* (from 2.8% to 10.0%; p<0.001) and decrease in *Klebsiella spp.* (from 3.5% to 0.3%; p=0.004) over time.

2.3.3. In vitro antimicrobial susceptibility and resistance profile

The in vitro antimicrobial susceptibilities for cephalosporin, fluoroquinolone, and aminoglycoside were 100.0% (25/25), 91.9% (205/223) and 95.2% (177/186) for Gram-positive bacteria; and 81.3% (65/80), 98.1% (212/216) and 98.3% (174/177) for Gram-negative bacteria (**Table 2.2**). From 2007-2013 to 2014-2019, there was an increase in resistance against penicillin in Gram-positive (from 3.5% to 12.7%; p=0.005) and Gram-negative bacteria (from 52.6% to 65.4%; p=0.22). There were only four (0.3%) MDR isolates and one (0.07%) MRSA noted in this study. The first-line treatment, either with combined therapy (cephalosporin and aminoglycoside) or fluoroquinolone monotherapy, provided good antibiotic coverage for 97.3% (n=396/407) and 95.2% (n=418/439) of the cases, respectively.

Antibiotic susceptibility of the four most commonly isolated microorganisms of IK, including *P. aeruginosa, S. aureus, Streptococci spp.,* and coagulase-negative staphylococcus, is summarised in **Table 2.3**. All these organisms were generally susceptible (>90%) to the commonly used cephalosporin (i.e. cefuroxime), aminoglycosides, and fluoroquinolones used in this study.

Table 2.1. Summary of microbiological profiles of IK in Nottingham in 2007-2019.

Organisms	**2007 – 2019 N=572; N (%)**	**2007 – 2013 N=282; N (%)**	**2014 – 2019 N=290; N (%)**	**P-value***
Gram-positive	308 (53.8)	153 (54.3)	155 (53.4)	0.53
S. aureus	91 (15.9)	49 (17.4)	42 (14.5)	0.34
CoNS	75 (13.1)	39 (13.8)	36 (12.4)	0.64
Streptococci	77 (13.5)	37 (13.1)	40 (13.8)	0.74
Bacilli	63 (11.0)	28 (9.9)	35 (12.1)	0.35
Others[#]	2 (0.3)	0 (0.0)	2 (0.7)	0.50
Gram-negative	223 (39.0)	108 (38.3)	115 (39.7)	0.74
P. aeruginosa	135 (23.6)	67 (23.8)	68 (23.4)	0.66
Moraxella spp.	37 (6.5)	8 (2.8)	29 (10.0)	<u><0.001</u>
Klebsiella spp.	11 (1.9)	10 (3.5)	1 (0.3)	<u>0.004</u>
Others[$]	40 (7.0)	23 (8.2)	17 (5.9)	0.21
Fungi	17 (3.0)	10 (3.5)	7 (2.4)	0.43
Yeast	10 (1.7)	6 (2.1)	4 (1.4)	0.91
Filamentous	7 (1.2)	4 (1.4)	3 (1.0)	0.91
Acanthamoeba	24 (4.2)	11 (3.9)	13 (4.5)	0.72

CoNS = Coagulase-negative staphylococci

*Chi-square or Fisher exact test (if any variable was <5) was used to detect any significant changing trend of the microbiological profiles between 2017-2013 and 2014-2019. The analysis was performed at two levels; the first level evaluated the changes among Gram-positive and Gram-negative organisms, fungi and Acanthamoeba; and the second level examined the changes of the subtypes of the organisms within the four groups. Significant P-values (<0.05) are underlined.

[#]Others include *Enterococci spp.*

[$]Others Include *Achromobacter spp., Acinetobacter spp., Citrobacter koseri, Enterobacter spp., Kingella spp., Serratia marcescens, Haaemophilus spp., Proteus spp., Neisseria spp.,* and *Stenotrophomonas maltophilia*.

Table 2.2. Summary of antibiotic susceptibility of IK in Nottingham in 2007-2019.

Organisms	**2007 – 2019** **N (%)**	**2007 – 2013** **N (%)**	**2014 – 2019** **N (%)**	**P-value***
Gram-positive				
Penicillin[#]	260/283 (91.9)	136/141 (96.5)	124/142 (87.3)	0.005
Cefuroxime	25/25 (100.0)	17/17 (100.0)	8/8 (100.0)	1.0
Gentamicin	177/186 (95.2)	97/101 (96.0)	80/85 (94.1)	0.73
Ciprofloxacin	164/182 (90.1)	92/100 (92.0)	72/82 (87.8)	0.35
Levofloxacin	41/41 (100.0)	16/16 (100.0)	25/25 (100.0)	1.0
Gram-negative				
Penicillin[#]	36/80 (45.0)	18/38 (47.4)	18/52 (34.6)	0.22
Cefuroxime	65/80 (81.3)	30/38 (78.9)	35/42 (83.3)	0.62
Amikacin	172/174 (98.9)	91/92 (98.9)	81/82 (98.8)	1.0
Gentamicin	174/177 (98.3)	93/94 (98.9)	81/83 (97.6)	0.60
Ciprofloxacin	175/179 (97.8)	92/94 (97.9)	83/85 (97.6)	1.0
Levofloxacin	53/53 (100.0)	16/16 (100.0)	37/37 (100.0)	1.0

*Chi-square was performed to determine the significant difference between the two time periods. Significant p-value is underlined.

[#]Penicillin group includes penicillin, amoxicillin, and flucloxacillin.

Table 2.3. Antibiotic susceptibility of the four most common organisms of IK in Nottingham during 2007-2019.

Antibiotics	*P. aeruginosa* N (%)	*S. aureus* N (%)	*Streptococci spp.* N (%)	CoNS N (%)
Penicillin[#]	0/1 (0.0%)	89/90 (98.9)	68/71 (95.8)	61/73 (83.6)
Cefuroxime	-	17/17 (100.0)	4/4 (100.0)	-
Gentamicin*	133/134 (99.3)	90/90 (100.0)	4/4 (100.0)	68/73 (93.2)
Amikacin*	133/133 (100.0)	2/2 (100.0)	-	-
Ciprofloxacin**	133/134 (99.3)	82/90 (91.1)	-	66/73 (90.4)
Levofloxacin**	6/6 (100.0)	-	39/39 (100.0)	-

CoNS = Coagulase-negative staphylococcus

[#]Penicillin group includes penicillin, amoxicillin, and flucloxacillin.

*Gentamicin and amikacin are aminoglycosides and usually one or the other was tested.

**Ciprofloxacin and levofloxacin are fluoroquinolone and usually one or the other was tested.

The percentage shown refers to the antibiotic susceptibility rate of each microorganisms.

Rate of resistance is equivalent to 100% minus the antibiotic susceptibility rate.

2.4. Discussion

IK represents a major cause of corneal blindness worldwide, particularly in the developing countries. To date, this represents the third study in the UK that reported the incidence as well as the causative microorganisms and *in vitro* antibiotic susceptibility and resistance profiles of IK. More importantly, it is the only study that examined the incidence of IK in the UK over the past decade.

2.4.1. Incidence

Currently, there is limited literature reporting on the incidence of IK globally. This is mainly due to the fact that most studies reported the incidence/prevalence of corneal blindness without distinguishing the underlying causes such as infective, inflammatory, traumatic, degenerative and others (Ung et al., 2019b, Ting et al.,

2021j). In this study, a stable trend of IK in Nottingham, UK, was observed over the past decade (2007-2019), with an estimated incidence of 34.7 per 100,000 population/year. This figure is comparable to the incidence previously reported in Portsmouth, UK, which was 40.1-52.1 per 100,000 population/year during 1997-2006, and substantially higher than the rate reported in the West of Scotland, which was 3.6 per 100,000 population/year during 1995. Consistent with the literature, the incidence of IK observed in this study was considerably lower than the rate in developing countries such as India and Nepal, which was estimated at 1.1-8.0 per 1000 people (or 110-799 per 100,000 population/year) (Gonzales et al., 1996, Upadhyay et al., 2001). Such significant variation of the incidence is primarily related to the population-based risk factors such as agricultural industry, high-risk occupation (with increased risk to corneal trauma), poorer environmental and personal hygiene, lower level of education, and poorer access to sanitation and healthcare in the developing countries (Ting et al., 2021j).

It is noteworthy to mention that the reported incidence of IK in this study and some other studies are likely to be underestimated as it was based on patients with IK who had undergone corneal scraping (Seal et al., 1999). Corneal scraping is usually performed in patients with moderate/severe IK with sizeable infiltrate where adequate sampling was possible or in patients with mild IK where the clinical presentation was atypical. Based on the local departmental protocol of QMC, Nottingham, all patients with a corneal infiltrate of >1mm or those with atypical infection were subjected to corneal scraping. This means that patients with mild and typical IK were not included in this study. In addition, viral keratitis cases were not captured in this study as the majority of cases were treated based on the typical clinical appearance of dendritic ulcer without any microbiological investigation. Nonetheless, the relatively stable incidence of IK observed in this study during the past decade suggests that IK represents a relatively common and persistent burden in the UK.

2.4.2. Microbiological profiles

Causative microorganisms of IK are subjected to wide geographical variations across the world (Shah et al., 2011). A systematic review of 36 studies demonstrated that bacteria were the most common isolates in developed countries whereas fungi were most commonly reported in developing countries (Shah et al., 2011). The recent ACSIKS study, which was conducted in Asia and included over 6000 patients with IK, demonstrated that fungi were the most common group of causative microorganism in China and India whereas bacteria were the most common organism in developed countries such as Singapore (Khor et al., 2018). Another large study conducted in the Southern China similarly reported a predominance of fungal keratitis in the region (Lin et al., 2019). The variation of microorganisms is likely influenced by various factors, including the occupational risk of corneal trauma, agricultural industry, use of contact lens, national income, and others (Shah et al., 2011, Ting et al., 2021j).

In this study, Gram-positive bacteria was shown to be the most common group of microorganisms responsible for IK during the entire study period. This finding parallels the results of many other studies conducted in the UK (Tan et al., 2017, Ting et al., 2018, Tavassoli et al., 2019, Orlans et al., 2011) and other countries (Hernandez-Camarena et al., 2015, Cariello et al., 2011, Peng et al., 2018). Within the UK, several studies (Tan et al., 2017, Tavassoli et al., 2019, Ting et al., 2018) have observed that coagulase-negative staphylococcus was most commonly isolated, which was in contrast to this study where *Pseudomonas spp.* was the main causative organism. This could be related to the differences in contact lens wear in different population groups, a fact that was not explored in this study and some other studies (Tavassoli et al., 2019, Ting et al., 2018, Tan et al., 2017). Interestingly, this study observed a significant increase trend in Moraxella keratitis in the Nottingham region that was similar to other regions in the UK such as Sunderland (Ting et al., 2018) and Manchester (Tan et al., 2017), suggesting a potentially emerging endemic

issue within the UK. In addition, polymicrobial keratitis presents unique diagnostic and therapeutic challenges to the clinicians as the treatment outcome is often variable and the treatment course is prolonged (Ting et al., 2019c, Lim et al., 2013). A total of 4.3% cases were of polymicrobial keratitis in this study, which was lower than the rate reported in the literature (10-14%) (Tan et al., 2017, Ting et al., 2018, Lin et al., 2019). It would be interesting and clinically valuable to examine the clinical outcomes of these polymicrobial cases as evidence on this area remains scarce (Tu et al., 2009).

The culture positivity rate was shown to be 37.7%, which was comparable to some studies (Tan et al., 2017, Tavassoli et al., 2019, Hernandez-Camarena et al., 2015) but lower than the others (Ting et al., 2018, Orlans et al., 2011). Plausible explanations for the relatively low culture yield include possible use of antibiotic before the visit to hospital, inadequate sampling from the infected corneas, and a lower threshold for performing corneal scrapes in non-infective cases, including sterile corneal melt and marginal keratitis. For patients who were already on any antibiotics before the hospital visit, the local standard practice was to stop all the antibiotics for 24-48 hours before performing any corneal scrapes. Therefore, it is likely that any prior use of antibiotics would have lesser impact than expected on the culture yield.

2.4.3. Antibiotic susceptibility and resistance

Antimicrobial resistance (AMR) is emerging as a global health threat of 21st century. AMR has been increasingly reported in both systemic and ocular infections (Prestinaci et al., 2015, Ung et al., 2019b). In this study, a substantial increase in penicillin resistance in both Gram-positive (12.7%) and Gram-negative bacteria (65.4%) was observed. However, most of the bacterial isolates were susceptible to the current broad-spectrum antibiotics (i.e. cephalosporin/aminoglycoside dual

therapy and fluoroquinolone monotherapy), which was similarly reported in other parts of the UK (Tan et al., 2017, Tavassoli et al., 2019). Reassuringly, there were only four (0.3%) MDR isolates and one (0.07%) MRSA identified.

Nonetheless, AMR in relation to IK is emerging as a serious concern in other parts of the world, including China (Lin et al., 2019), USA (Peng et al., 2018), and India (Oldenburg et al., 2013, Lalitha et al., 2017). For instance, the rate of MRSA ocular isolates was reported to be in the range of 0.1-5.0% in the UK (Tan et al., 2017, Tavassoli et al., 2019, Ting et al., 2018) whereas Antibiotic Resistance Monitoring in Ocular micRoorganisms (ARMOR) study conducted in the USA reported a substantially higher rate (36.6%) of MRSA ocular isolates (Thomas et al., 2019). Peng et al. (2018) observed that 35% of the ocular isolates were resistant to moxifloxacin and the rate increased over time. Similarly, Oldenburg et al. (2013) and Lalitha et al. (2017) reported a significant increase in fluoroquinolone (ofloxacin / moxifloxacin) resistance among *S. aureus* and *P. aeruginosa* isolated in South India. In addition, there was a significant increase in the number of MRSA from 2002 to 2013 in the same region (Lalitha et al., 2017). The discrepancy in the AMR rate in ocular isolates observed among different regions may be related to the difference in the prescribing practice (e.g. inappropriate and overuse of chloramphenicol eye drops for non-bacterial eye infection), choice of antibiotics used, environmental transmission, and genomic variations in the causative microorganisms.

2.4.4. Strengths and limitations

This study provides an up-to-date examination on the incidence of IK in one region of the UK. However, the incidence was calculated based on the number of IK cases that had corneal scrapings performed, thereby the incidence was likely underestimated. A prospective study with inclusion of all presumed IK, including those without corneal

scraping, could help ascertain the incidence of IK in the future. In addition, the true representation of the causative microorganisms is currently challenged by the low-to-moderate yield of the conventional microbiological investigation such as corneal scraping. Although the culture positivity rate (37.7%) was comparable to some studies, the moderate diagnostic yield highlights the need for further improvement (Ung et al., 2019b). This issue can be potentially ameliorated by other emerging investigative techniques such as *in vivo* confocal microscopy (Chidambaram et al., 2018a, Ting et al., 2019g), polymerase chain reaction (PCR) and/or next generation sequencing (Shimizu et al., 2019, Ung et al., 2020a), which have demonstrated their values in the diagnosis and clinical decision making in challenging IK cases. Current broad-spectrum antibiotics provide good treatment coverage in most IK cases; however, not all the antibiotics used were subjected to antibiotic susceptibility testing. As the commonly used topical antibiotics in ophthalmology differs from other specialties, a close collaboration with the microbiology department to standardise the *in vitro* antimicrobial susceptibility testing for IK would provide a more comprehensive evaluation of the susceptibility and resistance profiles.

In conclusion, IK represents a relatively common and persistent burden in the UK and the reported incidence is likely to be underestimated. Current broad-spectrum antimicrobial treatment provides good coverage for IK, albeit challenged by some level of AMR and polymicrobial infection. Future surveillance of the incidence, causative microorganisms, and antimicrobial susceptibility resistance with well-designed prospective studies would be beneficial.

CHAPTER 3

Seasonal Patterns of Incidence, Demographic Factors, and Microbiological Profiles of Infectious Keratitis: The Nottingham Infectious Keratitis Study

3.1. Introduction

Infectious keratitis (IK) is a common ophthalmic emergency characterised by a variety of manifestations, including corneal ulceration, stromal infiltrates and varying degree of anterior chamber reaction. It is primarily diagnosed on clinical grounds with the support of microbiological investigations, commonly in the form of corneal scraping for microscopy, culture and sensitivity testing. However, this current diagnostic approach is challenged by several issues, including the variably low culture yield, the slow turnaround time for positive results (usually 24-48 hours from the corneal samples being taken), contamination, and the possibility of polymicrobial infection (Fernandes et al., 2015, Ting et al., 2019c, Ung et al., 2019b). As the specific cause of IK is often indistinguishable from the clinical features, gaining knowledge about the patterns of microbiological profiles of IK in a particular region may provide additional guidance to the clinicians on the antimicrobial therapy.

Geographical and temporal variations of IK have been well reported in the literature, with bacteria and fungi being shown as the most common microorganisms responsible for IK in developed and developing countries, respectively (Shah et al., 2011, Khor et al., 2018, Ting et al., 2018, Ting et al., 2021h). However, examination of the seasonal trends in the incidence and causative microorganisms of IK remains

Table 3.1. Summary of the seasonal trends in the rate and microbiological profiles of infectious keratitis in the literature, in the order of chronology.

Year	Authors	Study period	Sample size*	Location	Overall seasonal rate	Microbiological profiles**
2008	Green et al.[10]	1999 – 2004	253	Brisbane, Australia	Not examined	*P. aeruginosa* (in summer); *S. pneumonia* (in winter)
2009	Ibrahim et al.[11]	1997 – 2003	1786	Portsmouth, UK	Summer > Winter > Autumn > Spring	Not examined
2012	Lin et al.[12]	2006 – 2009	6967	Southeast India	Summer > Winter > Spring / Autumn	Fungi (in summer); *P. aeruginosa* (in July-December)
2013	Otri et al.[13]	2007 – 2010	129	Nottingham, UK	Summer > Spring > Winter > Autumn	Not examined
2015	Ni et al.[14]	2009 – 2012	313	Philadelphia, US	Spring > Autumn > Summer > Winter	Bacteria (in spring)
2016	Gorski et al.[15]	2008 – 2013	155	New York, US	Summer > Winter > Spring > Autumn	*P. aeruginosa* (in summer)
2018#	Walkden et al.[16]	2004 – 2015	4229	Manchester, UK	Winter > Autumn > Spring > Summer	*P. aeruginosa* (in summer); CoNS (in autumn); Candida (in summer)
2020	Ting et al. (current study)	2008 – 2019	1272	Nottingham, UK	Summer > Autumn > Winter > Spring	*P. aeruginosa* (in summer); Gram-positive bacilli (in summer)

*Number of cases of infectious keratitis.

**Causative microorganisms which demonstrated significant seasonal predilection.

#The reported seasonal rate refers to the culture positivity rate of infectious keratitis but not the overall rate of infectious keratitis.

limited, particularly in the UK (**Table 3.1**) (Green et al., 2008, Ibrahim et al., 2009, Lin et al., 2012, Otri et al., 2013, Ni et al., 2015, Gorski et al., 2016, Walkden et al., 2018). So far, there are only three studies in the literature that examined the seasonal variations in the rate of IK in the UK (Walkden et al., 2018, Otri et al., 2013, Ibrahim et al., 2009). Otri et al. (2013) previously reported a higher proportion of IK during the summer season in Nottingham between 2007 and 2010; however only 129 cases of sight-threatening IK were included in the study. In addition, only one UK study, conducted in Manchester, examined the seasonal variations in the causative microorganisms of IK (Walkden et al., 2018).

In view of the paucity of literature, this study aimed to provide an up-to-date and comprehensive examination of the seasonal variations in the incidence, demographic factors, culture positivity rate, microbiological profiles and antibiotic susceptibility of IK in Nottingham.

3.2. Materials and methods

3.1.1. Case identification and data collection

This was a retrospective study of all patients who were diagnosed with IK and underwent corneal scraping between January 2008 and December 2019 (a 12-year period) at the Queen's Medical Centre (QMC), Nottingham, UK. The study method used was similar to the previous study (in **Chapter 2**) but with a different objective and a slightly different study period (Ting et al., 2021h). Cases were identified through the local microbiology electronic database. Based on the departmental guideline for IK, all patients presenting with sight threatening corneal ulcers (defined as size >1 mm diameter, central location, associated melting or hypopyon or atypical presentation) were subjected to microbiological investigation, which included corneal scraping for microscopy (with Gram staining), microbial culture and sensitivity testing

(Ting et al., 2021h). Corneal scraping was repeated in the same eye when the patient was unresponsive to treatment regardless of positive or negative outcome of the first culture. These cases were only counted as one clinical episode.

For descriptive and analytic purposes, the causative microorganisms were categorised into Gram-positive and Gram-negative bacteria, fungi, and Acanthamoeba. Seasons were divided into winter (22 December to 21 March), spring (22 March to 21 June), summer (22 June to 21 September), and autumn (22 September to 21 December), as defined by the internationally recognised astronomical seasons and previous studies (Ni et al., 2015, Chew et al., 2011, Gorski et al., 2016). The study was also divided into two time periods, namely 2008-2013 and 2014-2019, to examine for any temporal variation in the seasonal pattern of incidence of IK. The population in Nottingham was estimated at the range between 300,000 and 328,000 people during the study period (https://www.ukpopulation.org/nottingham-population/). These figures were used to estimate the incidence of IK in Nottingham, UK.

The study was conducted in accordance with the tenets of Declaration of Helsinki and was approved by the Nottingham University Hospitals NHS Trust as a service evaluation study (reference number: 19-265C).

3.1.2. Statistical analysis

Statistical analysis was performed using SPSS version 26.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). Chi-square test or one-way analysis of variance (ANOVA) was performed, where appropriate, to analyse the seasonal patterns of incidence, demographic factors, and microbiological profiles of IK among the four seasons. All continuous data were presented as mean ± standard deviation (SD)

and/or 95% confidence interval (CI). Pearson's correlation coefficient (*r*) analysis was performed to examine the incidence of IK in each season over time and was interpreted as weak (*r*=0.00-0.40), moderate (*r*=0.41-0.69), or strong (*r*=0.70-1.00), with negative values being interpreted in the same way (Ting et al., 2021l). P-value of ≤0.05 was considered statistically significant. When multiple subgroups were analysed in Chi-square test, crude Bonferroni-type adjustment was used to keep the overall false positive rate or alpha level at 0.05 [e.g. if comparison of 5 subgroups was performed, the adjusted p-value of ≤0.01 (based on 0.05/5) was considered significant] (Armstrong, 2014).

3.3. Results

3.3.1. Overall description

During the 12-year study period, a total of 1272 corneal scrapes were included. The mean patient's age was 50.0 ± 22.2 years and 50.2% were male. Of all corneal scrapes, 468 (36.8%) cases were culture positive with 549 microorganisms being identified (**Table 3.2**).

3.3.2. Seasonal changes in incidence of IK

The overall incidence of IK (in per 100,000 population-year) was highest during summer (37.7, 95% CI: 31.3-44.1), followed by autumn (36.7, 95% CI: 31.0-42.4), winter (36.4, 95% CI: 32.1-40.8), and spring (30.6, 95% CI: 26.8-34.3), though the overall difference was not statistically significant (p=0.14; **Figure 3.1**). Over the 12-year study period, there was a significant yearly increase in the incidence of IK during summer (r=0.58, p=0.049), but the incidence of IK in other seasons remained stable over time (**Figure 3.2**).

Table 3.2.Summary of the seasonal patterns of IK in Nottingham in 2008-2019.

	Winter N (%)	Spring N (%)	Summer N (%)	Autumn N (%)	P-value*
Age, years	52.4 ± 22.6	51.1 ± 22.5	48.3 ± 22.2	48.4 ± 21.5	0.044
Gender					0.88
Female	163 (49.7)	138 (50.2)	174 (51.3)	159 (48.2)	
Male	165 (50.3)	137 (49.8)	165 (48.7)	171 (51.8)	
Culture result					0.69
Positive	114 (34.8)	101 (36.7)	133 (39.2)	120 (36.4)	
Negative	214 (65.2)	174 (63.3)	206 (60.8)	210 (63.6)	
Organisms**					
Gram-positive	70 (54.7)	73 (60.3)	77 (49.7)	78 (53.8)	0.37
Staphylococci	46 (35.9)	38 (31.4)	35 (22.5)	40 (27.6)	0.055
Streptococci[#]	18 (14.1)	17 (14.0)	17 (11.0)	24 (16.6)	0.50
Bacilli	6 (4.7)	18 (14.9)	25 (16.1)	14 (9.7)	0.014
Gram-negative	48 (37.5)	38 (31.4)	70 (45.2)	55 (37.9)	0.14
PA	19 (14.8)	17 (14.0)	51 (32.9)	38 (26.2)	<0.001
Non-PA	29 (22.7)	21 (17.4)	19 (12.2)	17 (11.7)	0.036
Fungi	4 (3.1)	4 (3.3)	4 (2.6)	5 (3.4)	0.98
Acanthamoeba	6 (4.7)	6 (5.0)	4 (2.6)	7 (4.8)	0.70
Antibiotics, %***					
Cephalosporin	81.8 (27/6)	90.9 (20/2)	85.2 (23/4)	90.0 (18/2)	0.75
Aminoglycoside	97.5 (79/2)	92.6 (63/5)	97.2 (104/3)	97.7 (86/2)	0.28
Fluoroquinolone	92.4 (97/8)	93.2 (82/6)	96.6 (115/4)	97.2 (104/3)	0.27

PA = *Pseudomonas aeruginosa*

Continuous values are presented in mean ± standard deviation.

*Comparison was made among the four seasons using chi-square test or ANOVA test, where appropriate. P-value of ≤0.05 was considered statistically significant.

**Included all culture positive cases only and some cases cultured more than 1 organism. Comparison of organisms among 4 seasons was performed; (1) first level examining the 4 main groups, namely Gram-positive and Gram-negative bacteria, fungi and acanthamoeba; and (2) second level examining only the difference in the 5 bacterial subgroups.

[#]Included two cases of *Enterococcus faecalis* (one in spring and one in summer).

***Refers to antibiotic susceptibility, presented in % of susceptibility (Y=susceptible/N=resistant). The total number may vary as not all organisms were tested against all 3 classes of antibiotics.

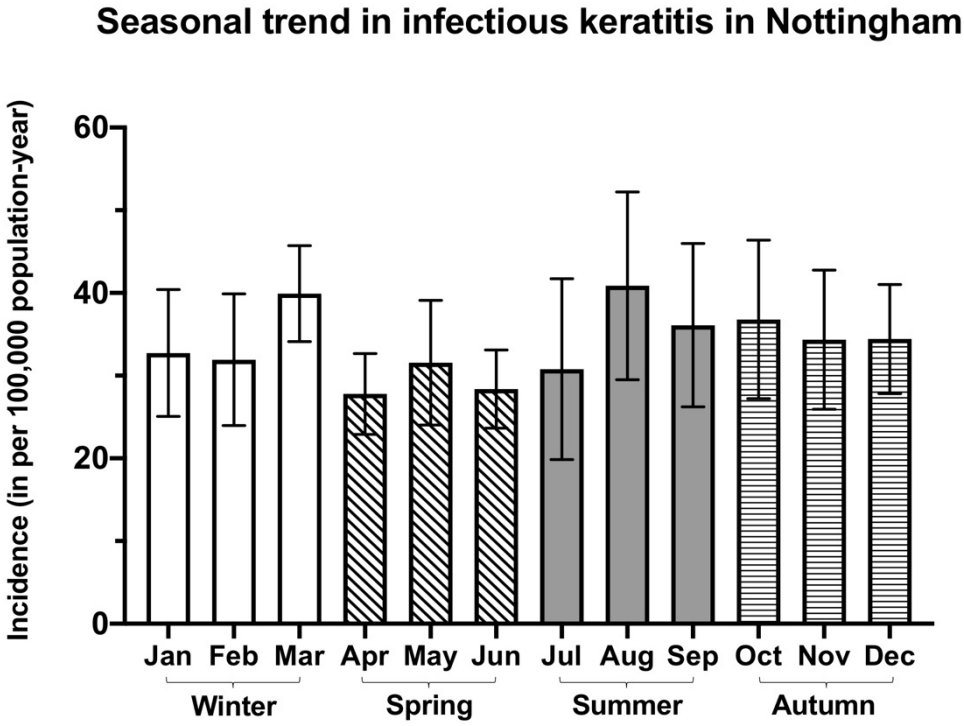


Figure 3.1. Seasonal patterns in the incidence of IK in Nottingham in 2008-2019.
The monthly incidence is presented as mean with 95% confidence interval (depicted by the error bars). For better graphical presentation purpose, “22 Dec – 21 Jan” was referred to as month “January”, “22 Jan – 21 Feb” was referred to as month “February”, and so on.

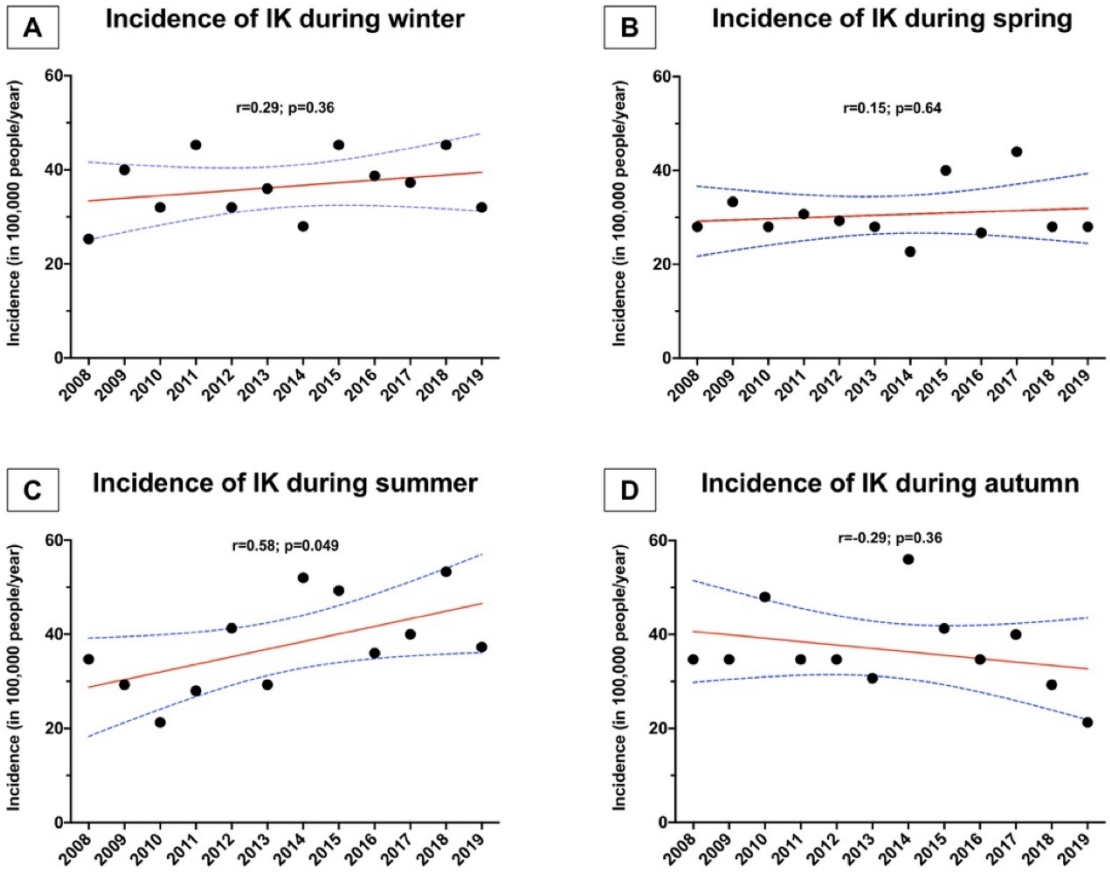


Figure 3.2. Temporal changes of the seasonal incidence of IK in Nottingham.

3.3.3. Seasonal patterns of demographic factors and microbiological profiles of IK

A total of 549 causative microorganisms were identified during the study period. There was a small but significant difference in the patient's age among the four seasons (p=0.044), with a younger group of patients (48.3 ± 22.2 years) presenting during the summer and older group of patients (52.4 ± 22.6 years) presenting during the winter (**Table 3.2**). In addition, seasonal predilection was observed in some causative organisms such as *P. aeruginosa* (32.9% in summer vs. 14.8% in winter; p<0.001) and Gram-positive bacilli (16.1% in summer vs. 4.7% in winter; p=0.014), which included *Propionibacterium spp.*, *Corynebacterium spp.*, and *Bacillus spp.* **Table 3.2**). There were no seasonal variations in gender, culture positivity rate, and antibiotic susceptibility of IK demonstrated among the four seasons.

3.4. Discussion

Seasonal cyclicity is a common feature of infectious diseases in general (Martinez, 2018). Depending on the causative pathogens, geographical and temporal factors, and host susceptibility, certain diseases are more common in particular seasons (Dowell, 2001, Martinez, 2018). For instance, influenza and rotavirus-related gastroenteritis were shown to be more common during the winter season (in temperate zones) (Cook et al., 1990, Martinez, 2018) whereas tuberculosis peaked during summer in some countries such as the UK (Cook et al., 1990, Koh et al., 2013). Therefore, understanding of the seasonal patterns of infectious diseases, including IK, could have important implications on the public health, disease control and biology (Martinez, 2018).

To date, this represents the most up-to-date and largest study examining the seasonal variations in incidence, demographic factors, and microbiological profiles of

IK in Nottingham, UK. This study observed that IK was most prevalent during summer (37.7 per 100,000 population-year), accompanied by a significant increase over the past decade. This was similar to other studies conducted in the UK (Ibrahim et al., 2009, Otri et al., 2013) and in other parts of the world such as India (Lin et al., 2012) and the US (Gorski et al., 2016), which also reported and a higher rate of IK during summer. Gaining knowledge on the seasonal rate or incidence of IK help increase the vigilance for IK among clinicians, including ophthalmologists and non-ophthalmologists who work at the front-line service such as accident and emergency department and primary care setting, during the prevalent season.

Plausible explanations for this seasonal phenomenon include raised temperature which may help the microorganisms to flourish, increased outdoor activities, contact with water, and use of contact lenses during the summer period, which could increase the risk of corneal injury and infection (Gorski et al., 2016). However, further studies examining the seasonal variations of the risk factors are required to elucidate the findings observed in this study. Interestingly, Walkden et al. (2018) reported that the culture positivity rate of IK was highest during winter and lowest during summer but it is uncertain whether the overall seasonal incidence of IK in their region could be inferred from these findings.

In addition, this study observed significant seasonal variations in *P. aeruginosa* and Gram-positive bacilli during the past decade. *P. aeruginosa* infection was most commonly observed during summer and was responsible for 33% of all IK. Similarly, a higher rate of *P. aeruginosa infection* in summer has been reported in other studies (Green et al., 2008, Lin et al., 2012, Gorski et al., 2016, Walkden et al., 2018), which was attributed to warmer temperature and use of contact lens. This study also demonstrated a significantly higher proportion of Gram-positive bacilli infection during summer when compared to winter. Gram-positive bacilli, including *Propionibacterium*

spp. and *Corynebacterium spp.*, are common ocular surface commensals (Suzuki et al., 2020, Zhang et al., 2017a) and the growth has been shown to be most active or optimal at the temperature between 30-37°C (Achermann et al., 2014), which may account for the higher rate of these infections during summer. Furthermore, Lin et al. (2012) have also demonstrated a significantly higher rate of fungal infection during summer in Southeast India. The number of fungal or Acanthamoeba infection were very low (<5%) in this study and no seasonal variation was observed.

Interestingly, studies have also shown that postoperative infection may be higher during summer. For instance, Anthony et al. (2018) demonstrated that surgical site infections following knee and hip arthroplasty were most common in summer, with increased re-admission for treatment of post-surgical infection during the same season. It would be interesting to examine whether this observation can be generalised to IK following ocular surface and/or refractive surgeries, particularly this study found that there was a significant higher risk of infection related to ocular surface commensals (i.e. *Propionibacterium spp.* and *Corynebacterium spp.*) during the summer season.

One of the limitations of this study is that it only included IK cases that had undergone corneal scraping; therefore, the overall incidence of IK in this region is likely to be underestimated. Nevertheless, there was no seasonal disparity in the practice pattern (e.g. culture method or threshold for performing corneal scraping) in QMC, Nottingham, suggesting that the findings related to the seasonal variations of IK observed in this study should not be affected. Another limitation is that the full representation of the causative microorganisms in this study was hindered by the relatively low positive culture rate, which is a common issue in many IK studies (Ung et al., 2019b, Ting et al., 2021j). While bacterial keratitis was shown to be the most common cause of IK (>90%) in this region, the relatively low incidence of fungal and

Acanthamoeba keratitis may be due to the inherent difficulty in identifying these organisms using conventional culture methods. Examining the medical case notes of the culture-negative cases, based on treatment response to various antimicrobial treatment and/or *in vivo* confocal microscopy (IVCM), may provide additional information on the potential responsible organisms for each particular case, which may include bacterial, fungal, Acanthamoeba, and polymicrobial infection. Emerging investigative techniques such as IVCM (Chidambaram et al., 2018a, Ting et al., 2019g), MALDI-TOF mass spectrometry (Singhal et al., 2015, Ting et al., 2020e), polymerase chain reaction (PCR) and/or next generation sequencing (Ung et al., 2020a), and artificial intelligence-assisted systems (Ting et al., 2021c, Rampat et al., 2021), could potentially enhance the diagnostic yield of IK and address the highlighted limitation in other similar studies in the future.

In conclusion, there has been a significant increase in IK during summer in Nottingham, UK, over the past decade. Increased awareness of IK during this season should be raised among the general public and the healthcare service. Gram-positive bacilli and *P. aeruginosa* infections are significantly more common in summer and these observations may provide additional guidance on the antimicrobial therapy used in the Nottingham region. Further studies investigating the correlations between these observations and the predisposing factors of IK will be beneficial.

CHAPTER 4

Risk Factors, Clinical Outcomes and Prognostic Factors of Bacterial Keratitis: The Nottingham Infectious Keratitis Study

4.1. Introduction

Infectious keratitis is a major cause of corneal blindness in both developed and developing countries (Ting et al., 2021j). The incidence has been estimated at 2.5-799 cases per 100,000 population/year (Ting et al., 2021j, Green et al., 2019a, Ting et al., 2021h). Subject to geographical, temporal and seasonal variations, bacteria and fungi are the most commonly implicated organisms in infectious keratitis (Ting et al., 2021h, Khor et al., 2018, Ting et al., 2021i, Lin et al., 2019, Shah et al., 2011). The variations are mainly attributed to the difference in the climate of the studied region and the population-based risk factors, particularly contact lens wear, trauma, and agricultural activities.

Bacterial keratitis (BK) has been consistently shown to be the main causative organisms in the UK and other developed countries. Based on the recent literature, BK represents 90-93% and 72-86% of all culture-positive infectious keratitis cases in the UK and in North America, respectively (Tan et al., 2017, Ting et al., 2018, Tavassoli et al., 2019, Ting et al., 2021h, Tam et al., 2017, Kowalski et al., 2020). In **Chapter 2**, it was shown that 92.8% of the culture-positive infectious keratitis cases were caused by bacteria, with *Pseudomonas aeruginosa* being the most common isolate (Ting et al., 2021h). In addition, in **Chapter 3**, a seasonal predilection of *P.*

aeruginosa keratitis in summer was observed, which has been hypothetically linked to the increased use of contact lens wear and trauma during outdoor/water activity (Ting et al., 2021i), though such associations remain to be elucidated.

In view of the prevalence of BK in the UK and other parts of the world, it is therefore important to understand the underlying risk factors (for preventative measures) and the clinical outcomes of BK. To date, the majority of UK studies had largely focussed on the epidemiology, causative microorganisms and antimicrobial resistance of bacteria (Tavassoli et al., 2019, Ting et al., 2021h, Ting et al., 2018, Tan et al., 2017), with limited information on the risk factors and outcomes of BK (Kaye et al., 2010, Otri et al., 2013). The aim of this study was to examine the risk factors, clinical characteristics, outcomes and prognostic factors of BK in Nottingham, UK. In addition, the clinical value of microbiological culture in the management of BK was examined as studies have shown that culture results may only be helpful in some, but not all, BK cases (McLeod et al., 1996, Ung et al., 2020b).

4.2. Materials and methods

This was a retrospective study of all cases of BK that presented to the Queen's Medical Centre, Nottingham, UK, between January 2015 and December 2019 (a 5-year period). The study was approved by the Clinical Governance team in the Nottingham University Hospitals NHS Trust as a Clinical Audit and Effectiveness Project (Ref: 19-265C).

4.2.1. Case identification and definition

Potential cases of BK were first identified via the local microbiological database as described in previous studies (Ting et al., 2021h, Ting et al., 2021i). Subsequently, the medical case records were examined to confirm the eligibility of the potential

cases prior to inclusion into the study. Both culture-positive and culture-negative presumed BK cases were included in this study. Culture-positive BK was defined as the presence of clinical BK with confirmation of the causative bacteria on microbiological culture. Culture-negative BK was diagnosed based on the clinical findings and the clinical course of the disease where improvement and/or resolution of the infection was achieved by intensive topical antibiotic treatment without other types of antimicrobial treatment. Cases that did not have complete initial and/or follow-up data were excluded from this study. In addition, culture-positive and culture-negative non-BK cases, including fungal, viral and parasitic keratitis, were excluded from the study.

4.2.2. Data collection

Relevant data, including demographic factors, risk factors, clinical characteristics, types of bacteria, corrected-distance-visual-acuity (CDVA), pre-existing ocular co-morbidities that could affect the visual prognosis, management, outcome and complications, were collected using a standardised excel proforma. Risk factors were divided into a number of categories, including: (1) contact lens wear; (2) trauma; (3) ocular surface diseases (e.g. dry eye, meibomian gland dysfunction, neurotrophic keratopathy, exposure keratopathy, previous corneal infection, recurrent corneal erosion syndrome, limbal stem cell deficiency, cicatricial conjunctivitis, band keratopathy, and bullous keratopathy); (4) use of topical corticosteroids; (5) previous or recent history of corneal surgery (e.g. corneal graft, pterygium surgery, corneal collagen cross-linking and corneal debridement / delamination); and (6) systemic immunosuppression (e.g. diabetes, use of systemic immunosuppressive drugs, malnutrition, and immunodeficiency). The size of epithelial defect and infiltrate were categorised as small (<3mm), moderate (3.1-6mm), or large (>6mm), based on the maximum linear dimension (**Figure 4.1**). The location of the ulcer was divided into

central (any part of the ulcer affecting the visual axis), paracentral (in between the central and peripheral location), and peripheral (the entire ulcer was within 3mm from the limbus; **Figure 4.1**). Recurrence was defined as the re-occurrence of BK after complete resolution of the previous BK episode, irrespective of the time interval between the first and subsequent infective episode. To avoid any duplication of the patient's risk factors in bilateral or recurrent BK cases, only one eye was included from each patient in this study. For recurrent cases, only the first BK episode was included and analysed, regardless of the laterality of infection in the subsequent infective episode.

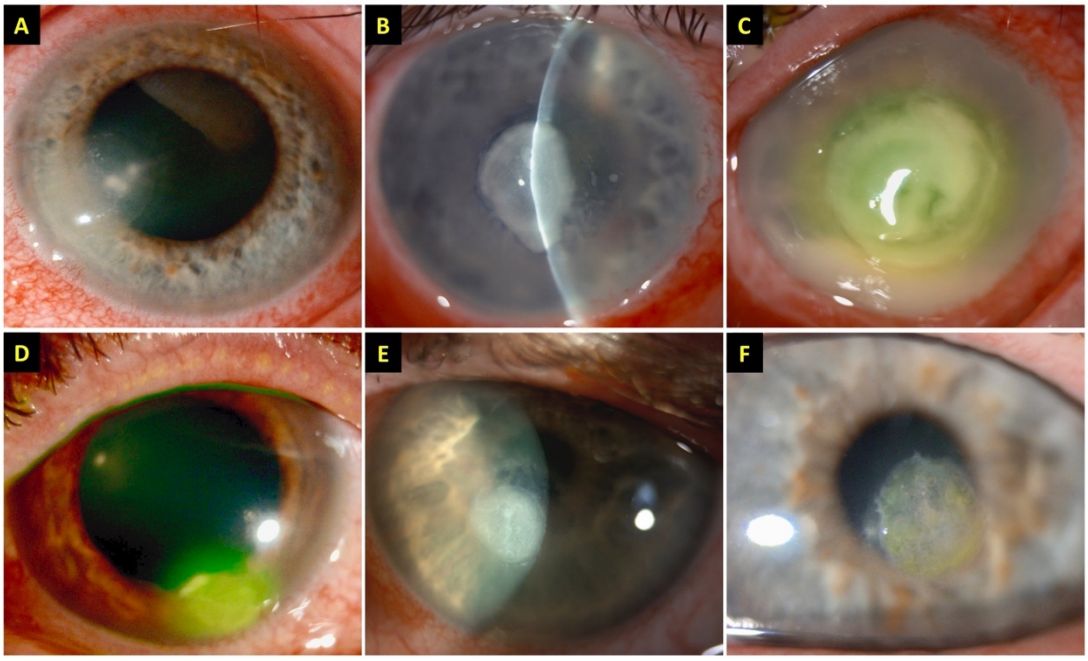


Figure 4.1. Examples of bacterial keratitis.
(A-C) Examples of bacterial keratitis with varying severity, including **(A)** small infiltrate (<3mm), **(B)** moderate infiltrate (3.1-6mm), and **(C)** large infiltrate (>6mm). **(D-F)** Examples of bacterial keratitis in different locations, including **(D)** peripheral, **(E)** paracentral, and **(F)** central location.

4.2.3. Microbiological culture, diagnosis and treatment

Based on the departmental guideline for infectious keratitis, all patients presented with corneal ulcer(s) of >1 mm diameter, central location or sight-threatening,

associated with significant anterior chamber reaction/hypopyon, or atypical presentation were subjected to microbiological investigation with corneal scraping for microscopy (with Gram staining), microbiological culture and sensitivity testing (Ting et al., 2021h, Ting et al., 2021i). Corneal scrapes were inoculated on chocolate agar (for fastidious organisms), blood agar (for bacteria), and Sabouraud dextrose agar (for fungi). For suspected cases of Acanthamoeba keratitis, corneal swab and/or epithelial biopsy was obtained for culture on non-nutrient agar with *Escherichia coli* overlay. All cultures were incubated for at least 1 week (and up to 3 weeks for suspected Acanthamoeba keratitis and fungal keratitis). The identity of microorganisms was confirmed through standard culture and bacteriology tests. In vivo confocal microscopy (IVCM) using the Heidelberg Retinal Tomography (HRT) II with Rostock Cornea Module (Heidelberg Engineering Ltd, Hertfordshire, UK) was utilised to aid the diagnosis (or exclusion) of fungal and Acanthamoeba keratitis.

All patients with BK were started on hourly topical treatment using either levofloxacin 0.5% monotherapy or combined therapy of fortified cephalosporin (cefuroxime 5%) and aminoglycoside (either amikacin 2.5% or gentamicin 1.5%), based on the severity of cases and the clinician's preference. Hospitalisation was warranted if the ulcer was severe (i.e. central, infiltrate >2mm, or presence of hypopyon) or was unresponsive to the initial antibiotic treatment, or the patient was unable or unlikely to comply with the intensive treatment regimen. All patients that were admitted for treatment were started on the combined therapy. Further changes to the antibiotic treatment were made, if necessary, based on the clinical course and the microbiological results. In cases with positive clinical response and progress, the patients were maintained on the same antibiotic regimen irrespective of the culture and sensitivity results.

4.2.1. Statistical analysis

Statistical analysis was performed using SPSS version 26.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). For descriptive and analytic purposes, the cases were divided into culture-positive and culture-negative BK cases. Comparison between groups was conducted using Pearson's Chi square or Fisher's Exact test where appropriate for categorical variables, and T test or Mann-Whitney U test for continuous variables. Normality of data distribution was assumed if the skewness and kurtosis z-values were between -1.96 and +1.96 and the Shapiro-Wilk test p-value was >0.05. All continuous data were presented as mean ± standard deviation (SD) and/or 95% confidence interval (CI).

The main outcome measures were corrected-distance-visual-acuity (CDVA) and time to complete corneal healing, defined as complete resolution of infection with corneal re-epithelialisation. Snellen vision was converted to logMAR vision for analytic purpose. Vision of counting fingers (CF), hand movement (HM), perception of light (PL) and no perception of light (NPL) were quantified as 1.9 logMAR, 2.3 logMAR, 2.8 logMAR and 3.0 logMAR respectively (Lange et al., 2009, Grinton et al., 2021). For cases that required therapeutic or tectonic keratoplasty, the vision prior to the transplant was used as the final CDVA. In enucleation or evisceration cases, a CDVA of 3.0 logMAR (equivalent to NPL vision) was assigned as the final vision. Multivariable logistic regression analysis was performed to examine for any potential prognostic factors for poor visual outcome, defined as CDVA of worse than 6/24 (or <0.6 logMAR), and poor corneal healing, defined as >30 days to achieve complete corneal healing, occurrence of uncontrolled infection or corneal perforation requiring corneal gluing, tectonic or therapeutic keratoplasty, and/or evisceration / enucleation. The results of logistic regression analyses were presented in odd ratios (ORs) with 95% confidence interval (CI). P-value of <0.05 was considered statistically significant.

4.3. Results

4.3.1. Overall description

During the 5-year study period, a total of 283 patients (n = 283 eyes) with BK were included. The mean age was 54.4 ± 21.0 years (range, 4.9-92.7 years), 50.9% patients were male, and 51.2% cases affected the left eye (**Table 4.1**). Two bilateral BK cases were identified and only the right eye was included. The mean follow-up duration was 6.0 ± 8.9 months. Of all included cases, 128 (45.2%) and 155 (54.8%) cases were culture-positive and culture-negative BK (**Table 4.1)**.

4.3.2. Risk factors and causative organisms

Nearly all (273, 96.5%) patients were found to have at least one risk factor, with 66 (23.3%) patients having two risk factors, and 18 (6.4%) patients having three or more risk factors for BK. Ocular surface diseases (134, 47.3%) were the most common risk factor, followed by contact lens wear (100, 35.3%), systemic immunosuppression (52, 18.4%), prior corneal surgery (39, 13.8%), use of topical corticosteroids at presentation (31, 11.0%), and trauma (25, 8.8%; **Table 4.1**). Contact lens wear was more commonly associated with younger patients (≤50 years) whereas systemic immunosuppression was more commonly associated with older patients (>50 years; **Table 4.2**).

Of the 128 culture-positive cases, 10 (7.8%) cases grew more than one species, with a total of 138 bacteria being identified (**Table 4.3**). *Pseudomonas aeruginosa* (44, 31.9%) was the most common isolate identified, followed by *Staphylococci spp.* (36, 26.1%) and *Streptococci spp.* (16, 11.6%). Contact lens wear was most commonly associated with Gram-negative bacteria (30, 66.7%), *P. aeruginosa* (23, 51.1%), whereas Gram-positive bacteria, particularly *Staphylococci spp.*, was most commonly implicated in non-contact lens-related BK cases, including those affected by ocular

surface disease (38, 60.3%), use of topical corticosteroids (16, 64%), and previous history of corneal surgery (13, 61.9%; p=0.017; **Table 4.3**).

4.3.3. Clinical characteristics

The baseline clinical characteristics are summarised in **Table 4.1**. At baseline, 124 (43.8%) patients presented with a CDVA of <1.0 logMAR. The most commonly observed clinical characteristics of the ulcer were small epithelial defect size (172, 60.8%), small infiltrate size (183, 64.7%), central location (110, 38.9%), and absence of hypopyon (201, 71.0%). The mean duration of symptoms prior to presentation was 6.0 ± 13.0 days. Hospitalisation for intensive treatment was required in 162 (57.2%) patients, with a mean hospitalisation duration of 8.0 ± 8.3 days. The baseline clinical characteristics of BK were significantly different between culture-positive and culture-negative cases. Culture-positive cases were more commonly associated with older age (p=0.004), prior corneal surgery (p=0.011), use of topical corticosteroids (p=0.008), poorer presenting CDVA (p<0.001), larger epithelial defect / infiltrate size (p<0.001), central or paracentral ulcer (p=0.002), presence of hypopyon (p<0.001), and need for hospitalisation for intensive treatment (p<0.001).

4.3.4. Medical and surgical treatment

At initial presentation, 150 (53.0%) patients and 133 (47.0%) patients were started on combined therapy and monotherapy, respectively. Of the 128 culture-positive cases, 51 (39.8%) cases (equivalent to 18.2% of all included cases) had the treatment plan altered due to the culture and sensitivity results and/or unsatisfactory clinical progress. A total of 237 (83.7%) patients were successfully treated with medical treatment alone, while 46 (16.3%) patients required additional surgical interventions for controlling the infection and/or its sequelae.

Table 4.1. Summary of bacterial keratitis in Nottingham, UK.

Parameters	**All cases Total N = 283; N (%)**	**CP; Total N = 128; N (%)**	**CN; Total N = 155; N (%)**	**P-value[#]**
Age, years	54.4 ± 21.0	58.5 ± 21.3	51.1 ± 20.1	0.004
Gender				0.66
Female	139 (49.1)	61 (47.7)	78 (50.3)	
Male	144 (50.9)	67 (52.3)	77 (49.7)	
Laterality				0.13
Left	145 (51.2)	72 (56.3)	73 (47.1)	
Right	138 (48.8)	56 (43.7)	82 (52.9)	
Risk factors[$]				0.030
OSD*	134 (47.3)	59 (46.1)	75 (48.4)	0.70
Contact lens wear	100 (35.3)	41 (32.0)	59 (38.1)	0.29
Immunosuppression**	52 (18.4)	27 (21.1)	25 (16.1)	0.28
Prior corneal surgery	39 (13.8)	25 (19.5)	14 (9.0)	0.011
Topical corticosteroids	31 (11.0)	21 (16.4)	10 (6.5)	0.008
Trauma	25 (8.8)	10 (7.8)	15 (9.7)	0.58
None identified	10 (3.5)	3 (2.3)	7 (4.5)	0.32
Presenting CDVA, in logMAR				<0.001
0.0 – 0.3	89 (31.4)	20 (15.6)	69 (44.5)	
<0.3 – 0.6	41 (14.5)	20 (15.6)	21 (13.5)	
<0.6-1.0	29 (10.2)	15 (11.7)	14 (9.0)	
<1.0	124 (43.8)	73 (57.0)	51 (32.9)	
Size of epithelial defect				<0.001
Small (≤3mm)	172 (60.8)	58 (45.3)	114 (73.5)	
Moderate (3.1-6mm)	63 (22.3)	43 (33.6)	20 (12.9)	
Large (>6mm)	48 (17.0)	27 (21.1)	21 (13.5)	
Size of infiltrate				<0.001
Small (≤3mm)	183 (64.7)	65 (50.8)	118 (76.1)	
Moderate (3.1-6mm)	62 (21.9)	40 (31.2)	22 (14.2)	
Large (>6mm)	38 (13.4)	23 (18.0)	15 (9.7)	
Location				0.002
Central	110 (38.9)	57 (44.5)	53 (34.2)	
Paracentral	106 (37.5)	53 (41.4)	53 (34.2)	
Peripheral	67 (23.7)	18 (14.1)	49 (31.6)	
Hypopyon				<0.001
Yes	82 (29.0)	60 (46.9)	22 (14.2)	
No	201 (71.0)	68 (53.1)	133 (85.8)	
Hospitalisation required				<0.001
Yes	162 (57.2)	95 (74.2)	67 (43.2)	
No	121 (42.8)	33 (25.8)	88 (56.8)	
Duration of hospitalisation, days	8.0 ± 8.3	8.8 ± 9.2	6.0 ± 4.9	0.06

OSD = Ocular surface disease; CDVA = Corrected-distance-visual-acuity
Continuous values are presented as mean ± standard deviation (SD).
[$]Some patients had more than 1 risk factor identified.
*Includes dry eye disease, meibomian gland disease, neurotrophic keratopathy, exposure keratopathy, previous corneal infection, corneal erosion syndrome, limbal stem cell deficiency, cicatricial conjunctivitis, band keratopathy, and bullous keratopathy.
**Includes diabetes, use of immunosuppressive drugs, malnutrition, and immunodeficiency.
[#]Comparison between culture-positive (CP) and culture-negative (CN) cases. Chi-square and unpaired T-test were used for categorical and continuous variables, respectively. Significant values are underlined.

Various surgical interventions were performed to maintain / restore the globe integrity and/or to promote corneal healing, with some patients requiring multiple surgical procedures. These included corneal gluing (22, 7.8%), temporary / permanent tarsorrhaphy (13, 4.6%), single or multi-layer amniotic membrane transplant (11, 3.9%), conjunctival hooding (3, 1.1%), and emergency therapeutic / tectonic keratoplasty (2, 0.7%), evisceration (2, 0.7%), and enucleation (2, 0.7%). Among all, 21 corneal gluing, 5 amniotic membrane transplant, 3 tarsorrhaphy, 1 tectonic keratoplasty, and 1 evisceration were warranted in cases with threatened / actual corneal perforation. Other procedures were performed to expedite corneal healing and eradicate the infection. Five (1.8%) patients required elective optical penetrating keratoplasty after the resolution of infection.

Table 4.2. Summary of risk factors based on different age groups.

Risk factors	**Age ≤ 50 years Total N = 118 N (%)**	**Age > 50 years Total N = 165 N (%)**	**P-value**
Presence of risk factors			0.66
None	4 (3.4)	6 (3.6)	
One	78 (66.1)	111 (67.3)	
Two	26 (22.0)	40 (24.2)	
Three or more	10 (8.5)	8 (4.8)	
Type of risk factors			<0.001
OSD*	54 (45.8)	80 (50.6)	0.65
Contact lens wear	68 (57.6)	32 (19.4)	<0.001
Immunosuppression**	9 (7.6)	43 (26.1)	<0.001
Prior corneal surgery	15 (12.7)	24 (14.5)	0.19
Topical corticosteroids	8 (6.8)	23 (14.6)	0.06
Trauma	10 (8.5)	15 (9.1)	0.86

OSD = Ocular surface disease

*Includes dry eye disease, meibomian gland disease, neurotrophic keratopathy, exposure keratopathy, previous corneal infection, corneal erosion syndrome, limbal stem cell deficiency, cicatricial conjunctivitis, band keratopathy, and bullous keratopathy.

**Includes diabetes, use of systemic immunosuppressive drugs, malnutrition, and immunodeficiency

Table 4.3. Summary of causative organisms and risk factors of bacterial keratitis in Nottingham, UK.

	Total* N=138 N (%)	OSD N=63 N (%)	CL wear N=45 N (%)	CS N=25 N (%)	TC N=21 N (%)	P-value**
Gram-positive	70 (50.7)	38 (60.3)	15 (33.3)	16 (64.0)	13 (61.9)	0.017
Staphylococci	36 (26.1)	20 (31.7)	9 (20.0)	10 (40.0)	7 (33.3)	
Streptococci	16 (11.6)	9 (14.3)	2 (4.4)	4 (16.0)	5 (23.8)	
Other GP	18 (13.0)	9 (14.3)	4 (8.9)	2 (8.0)	2 (9.5)	
Gram-negative	68 (49.3)	25 (39.7)	30 (66.7)	9 (36.0)	8 (38.1)	
Pseudomonas	44 (31.9)	13 (20.6)	23 (51.1)	6 (24.0)	4 (19.0)	
Moraxella	14 (10.1)	8 (12.7)	3 (6.7)	3 (12.0)	3 (14.3)	
Other GN	10 (7.2)	4 (6.3)	4 (8.9)	0 (0.0)	1 (4.8)	

OSD = Ocular surface disease; CL = Contact lens; TC = Topical corticosteroids; CS = Previous corneal surgery; GP = Gram-positive; GN = Gram-negative

*The total number of organisms exceeded the total number of culture-positive cases as some cases were polymicrobial. In addition, some cases had more than one risk factor identified, and the same implicated organism was included in more than one group of risk factor.

**Comparison of the causative organisms among different risk factors were performed. The analysis was performed at the level of Gram-positive versus Gram-negative bacteria only.

4.3.5. Clinical outcomes and prognostic factors

The mean CDVA (in logMAR) improved from 1.17 ± 1.03 at presentation to 0.80 ± 1.00 at final follow-up (p<0.001). From baseline to final follow-up, the proportion of patients with CDVA of ≥0.30 logMAR improved from 31.4% to 53.0%, with the proportion of CDVA of <1.0 logMAR reducing from 43.8% to 30.4% (p<0.001; **Figure 4.2**). Twenty-three (8.1%) patients had a final CDVA of PL or worse, including four (1.4%) patients that had evisceration / enucleation. Multivariable logistic regression demonstrated that poor visual outcome (CDVA <0.6 logMAR) was significantly influenced by age >50 years old (OR 2.61; 95% CI, 1.24-5.47; p=0.011), infiltrate size >3mm (OR 4.07; 95% CI, 1.21-13.73; p=0.024), central ulcer (OR 2.13; 95% CI, 1.01-4.51; p=0.047), and presenting CDVA of <0.6 logMAR (OR 29.70; 95% CI, 10.47-84.18; p<0.001; **Table 4.4**).

In terms of complete corneal healing, 278 (98.2%) patients achieved complete corneal healing at final follow-up, with four patients requiring evisceration / enucleation and one patient was still undergoing active treatment. The mean corneal healing time was 1.6 ± 1.5 months, with 157 (55.5%) patients had a corneal healing time of >30 days. Multivariable logistic regression analysis demonstrated that poor corneal healing (>30 days to achieve complete healing) was significantly affected by age >50 years old (OR 1.86; 95% CI, 1.06-3.24; p=0.030), involvement of right eye (OR 1.82; 95% CI, 1.05-3.16; p=0.033), infiltrate size >3mm (OR 3.46; 95% CI, 1.24-9.70; p=0.018), and presenting CDVA of <0.6 logMAR (OR 2.22; 95% CI, 1.19-4.15; p=0.013; **Table 4.4**). Other factors such as gender, culture positivity, and presence of hypopyon did not significantly influence the visual outcome or the corneal healing time (all p>0.05).

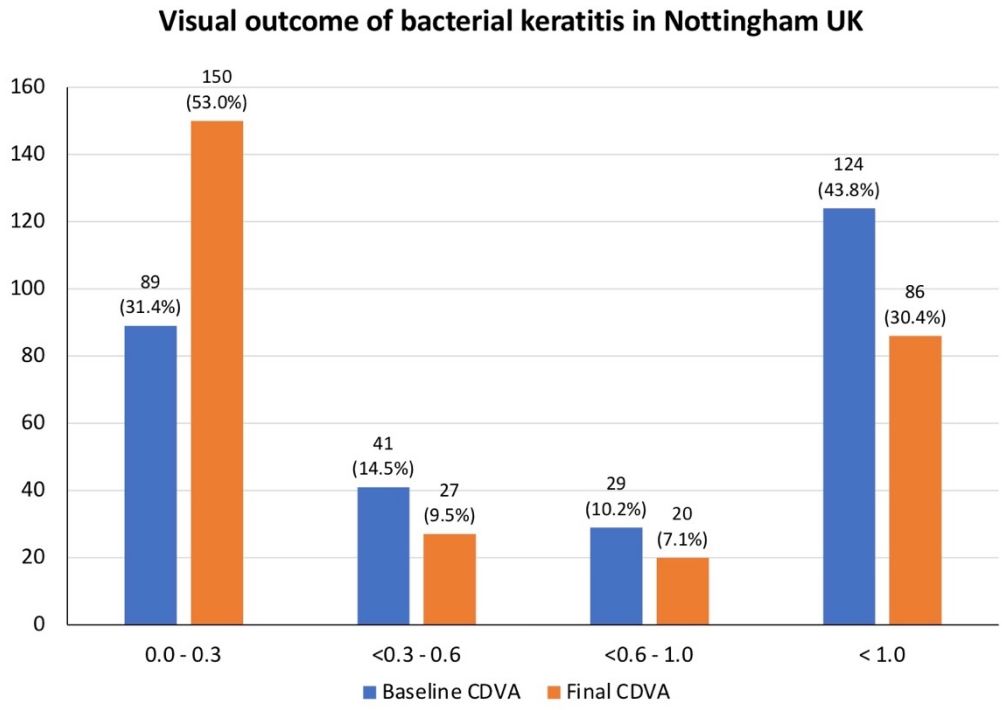


Figure 4.2. Visual outcome of bacterial keratitis in Nottingham.

Table 4.4. Prognostic factors for visual outcome and corneal healing in bacterial keratitis.

	Poor visual outcome[#]		Poor corneal healing[$]	
Parameters	OR (95% CI)	P-value*	OR (95% CI)	P-value*
Age > 50 years	2.61 (1.24 – 5.47)	0.011	1.86 (1.06 – 3.24)	0.030
Female gender	0.81 (0.41 – 1.62)	0.56	1.27 (0.73 – 2.19)	0.40
Right eye	0.81 (0.41 – 1.60)	0.54	1.82 (1.05 – 3.16)	0.033
Epithelial defect >3mm	0.69 (0.19 – 2.50)	0.57	1.23 (0.44 – 3.44)	0.69
Infiltrate size >3mm	4.07 (1.21 – 13.7)	0.024	3.46 (1.24 – 9.70)	0.018
Central ulcer	2.13 (1.01 – 4.51)	0.047	1.30 (0.67 – 2.54)	0.43
Presence of hypopyon	0.47 (0.21 – 1.08)	0.08	1.02 (0.50 – 2.07)	0.96
Positive culture results	1.17 (0.56 – 2.46)	0.67	1.15 (0.63 – 2.10)	0.65
Presenting CDVA <0.6	29.70 (10.5 -84.1)	<0.001	2.22 (1.19 – 4.15)	0.013

OR = Odd ratio

[#]Poor visual outcome was defined as corrected-distance-visual-acuity (CDVA) <0.60logMAR.

[$]Poor corneal healing was defined as >30 days to achieve complete healing or occurrence of corneal perforation or uncontrolled infection.

*Multivariable logistic regression analysis was performed. Significant p-values are underlined.

4.3.6. Complications

A number of complications were observed in this study, including raised intraocular pressure (>21 mmHg) / glaucoma (32, 11.3%), recurrence of infection (28, 9.9%), threatened / actual corneal perforation (25, 8.8%), complete loss of vision / NLP (11, 3.9%), and loss of eye (4, 1.4%), and phthisis bulbi (1, 0.4%).

4.4. Discussion

BK is the most common cause of infectious keratitis in the UK and in many developed countries. This study represents one of the largest and most up-to-date studies in the UK specifically examining the risk factors, clinical characteristics, outcomes and prognostic factors of BK.

4.4.1. Risk factors and causative organisms

BK rarely occurs in the absence of any predisposing factor. In this study, relevant risk factors were identified in 96.5% of the patients, with 29.7% of them having two or more risk factors. Identification of risk factors for BK is important as it allows the clinicians to manage the identified risk factors to reduce the risk of recurrence of infection and helps provide some insights into the underlying causative organisms, thereby guiding the choice of antimicrobial treatment, especially in the absence of positive microbiological culture results. This makes the treatment or control of risk factors that cannot be cured, very important in the prevention of BK.

Risk factors for infectious keratitis have been shown to vary considerably across different studies (Ting et al., 2021j). Contact lens wear is one of the most common risk factors for infectious keratitis in the developed countries whereas trauma is most commonly implicated in developing countries (Ting et al., 2021j, Chidambaram et al., 2018b, Khoo et al., 2020, Bourcier et al., 2003). Kaye et al. (2010) previously conducted a multi-center study in the UK examining the risk factors and outcomes of BK in 2003-2006. The most common risk factor was found to be corneal / ocular surface diseases (50%) and contact lens wear (32%). Another UK study conducted by Dua's group in 2007-2010 examining the profile of sight-threatening infectious keratitis in Nottingham (which included BK and Acanthamoeba keratitis) observed that ocular surface disease (33%), contact lens wear (26.5%) and previous ocular surgery (20.2%) were the most common risk factors. A similar distribution of the risk factors was observed in this study where ocular surface diseases and contact lens wear were found to be most common factors, suggesting that the population-based risk factors for BK in the UK had remained similar over the past two decades.

In contrast, Khoo et al. (2020) had recently examined the clinical characteristics of infectious keratitis (all types of organisms) in Sydney, Australia, and reported that

contact lens wear (63%) and topical use of steroid (24%) were the most common risk factors, highlighting the difference in risk factors among different regions, even in the setting of developed countries. Gaining a better knowledge of the region-specific population risk factors enables a more targeted preventative strategy and research focus for reducing the incidence and burden of infectious keratitis. Interestingly, an immunosuppressed state (which included diabetes) was found to be the third most common risk factors for BK in this study. This association might be attributed to the overall reduced immunity and specifically at the ocular surface, promotion of microbial growth (in hyperglycaemia), and presence of undiagnosed ocular surface diseases such as dry eye disease and neurotrophic keratopathy that are commonly linked to diabetes (Ting et al., 2021j, Li et al., 2019b, Jeng et al., 2010).

This study observed that contact lens-related BK was most commonly caused by *P. aeruginosa*, which is consistent with the findings of many other studies. This observation also provides support to the hypothesis of previous work on the increased prevalence of *P. aeruginosa*-related BK during the summer season due to increased contact lens wear (Ting et al., 2021i). On the other hand, Gram-positive bacteria, including *Staphylococci spp.*, which are common ocular surface commensals, are more frequently identified in BK cases affected by ocular surface diseases, prior ocular surgery and use of topical steroids. This was consistent with other studies whereby Gram-positive bacteria were most commonly implicated in BK associated with ocular surface diseases (Khoo et al., 2019). Therefore, in non-contact lens-related culture-negative BK cases that are not responsive to fluoroquinolone monotherapy, adding a cephalosporine would be beneficial as it normally provides good coverage to Gram-positive bacteria (Ting et al., 2021h).

4.4.2. Clinical characteristics

In this study, it was shown that many of the BK cases were of mild severity (i.e. small ulcer size without the presence of hypopyon). This was likely attributed to the fact that the majority of the patients sought medical attention within the first week of their ocular symptoms. This may also explain the lesser (16%) need for additional surgical interventions. On the other hand, an Indian study of infectious keratitis conducted two decades ago showed that only 0.02% of their patients presented within the first week of ocular symptoms, with 12% of the cohort presenting one month after the onset of symptoms. Notably, 43% of their BK patients required surgical interventions, considerably higher than this study. Another recent Indian study of infectious keratitis conducted at another region observed that the median duration of ocular symptoms was 7 days, with 72% cases caused by corneal trauma. The heterogeneity in the promptness of patients seeking medical attention is likely related to the difference in the culture, level of education and health awareness, causes / risk factors (earlier in trauma-related cases), and accessibility to healthcare facility. Although the analysis showed that patients with duration of ocular symptoms of ≥7 days had a worse visual outcome (<0.6 logMAR CDVA), the association was not significant in the multivariable regression analysis (not presented herein). The duration of ocular symptoms was not included as one of the independent variables in the current regression model in view of the high amount (~15%) of missing data in this parameter, which could negatively affect the multivariable regression analysis.

4.4.3. Microbiological culture and its clinical value

Corneal scraping for culture and sensitivity testing remains the most common microbiological investigations for infectious keratitis. While the culture yield has been shown to be variably low (23.7-77%) (Ting et al., 2021j, Peng et al., 2018, Kaliamurthy et al., 2013), this is currently the only method that could provide both the information

of the underlying causative organisms and the antimicrobial susceptibility and resistance results. In this study, 45% of the cases were culture-positive but this was not truly reflective of the culture yield of infectious keratitis in the studied region as cases with incomplete data or inconclusive cause were excluded from this study. This study observed that culture positivity was significantly associated with several factors, including increased age, large ulcer size, central ulcer, prior corneal surgery or use of topical steroids, and worse presenting vision. Such association is likely attributed to the more severe disease and higher microbial load at presentation. This is in accordance with the "1, 2, 3 Rule" advocated by Vital et al. that corneal culture should be performed when any of the three clinical parameters is met (i.e. ≥1+ anterior chamber cells, ≥2 mm infiltrate, or infiltrate ≤3 mm distance from the corneal center) as it predicts the severity, outcome and likelihood of positive culture in infectious keratitis (Ung et al., 2020b, Vital et al., 2007). In addition, Cariello et al. (2011) similarly showed that previous use of topical steroids increased the chance of positive culture. Therefore, these findings suggest that performing corneal culture in older patients with more severe disease and with prior use of topical steroids is more likely to have a positive culture and thence better clinical value.

McLeod et al. (1996) previously examined the role of microbiological investigations in managing infectious keratitis. All patients were treated with a fortified cefazoline and a fortified aminoglycoside. They found that all moderate BK resolved without any modification in the antibiotic treatment and 7% of the severe BK cases required a change in the treatment regimen. In this study, 39% of all culture-positive cases (or 18% of all included cases) were found to have the antibiotic treatment regimen altered due to the culture and sensitivity results. This underlines the clinical value of microbiological investigations, particularly in more severe cases, for managing infectious keratitis. The considerably higher rate of changes in treatment was partly related to the fact that some of the patients were only treated with levofloxacin at the

initial presentation. In addition, in cases where Gram-negative bacteria were cultured and the initial treatment response was slow or unsatisfactory, the treatment was switched to combined therapy consisting of a fortified aminoglycoside and a fluoroquinolone (if the organisms were susceptible to both antibiotics).

Furthermore, as this study only included BK cases, the value of positive microbiological culture may be underestimated. For instance, a case with an initial diagnosis of presumed BK that subsequently cultured non-bacterial organisms (e.g. fungi or Acanthamoeba) could lead to a change in antimicrobial treatment, though these cases were not included in this study. The same would apply to mixed infections.

4.4.4. Outcomes and prognostic factors

The majority (84%) of our cases healed with medical treatment alone. While 25 (9%) patients developed threatened / actual perforation, most of them (21, 84%) were amenable to corneal gluing, multi-layer amniotic membrane transplant or conjunctival hooding. This is in contrast with the findings of the Asia Cornea Society Infectious Keratitis Study (ASCIKS) whereby ~10% of the cohort required emergency therapeutic keratoplasty (Khor et al., 2018). Another Australian study, which included all types of infectious keratitis, showed that 6% of the patients required either therapeutic keratoplasty, evisceration or enucleation (Khoo et al., 2020). The discrepancy among the studies may be related to the difference in the severity of the presenting ulcer, the risk factors (lower proportion of trauma in this study), and the inclusion of fungal keratitis and/or polymicrobial keratitis, which are often more difficult to manage compared to BK (Khor et al., 2018, Gopinathan et al., 2009, Khoo et al., 2020, Chidambaram et al., 2018b, Ting et al., 2019c).

A number of important prognostic factors for visual outcome and corneal healing were identified in this study. This study observed that poor visual outcome was significantly influenced by older age, larger infiltrate, central ulcer, and poor presenting CDVA. Khoo et al. (2020) similarly demonstrated that older patients with worse presenting vision and larger ulcer were more likely to experience a poor outcome, which was defined as vision of <6/60, decrease vision during treatment, or occurrence of complications requiring keratoplasty, evisceration or enucleation. Parmar et al. (2006) also reported that elderly patients (≥65 years old) were more commonly affected by central and larger ulcers and worse visual outcome.

Additionally, this study showed that corneal healing was negatively affected by older age, larger infiltrate size and poorer presenting vision. Gaining a better knowledge of these prognostic factors may enable earlier interventions (e.g., temporary tarsorrhaphy or amniotic membrane transplant) to help promote corneal healing and re-epithelialisation after the acute sterilisation phase (Dua et al., 2004, Dua et al., 2018, Ting et al., 2021n). The poorer corneal wound healing in older patients with BK is likely related to the presence of co-existing ocular surface diseases (e.g. dry eyes, neurotrophic keratopathy, and others), immunosuppression, and the age-related reduction in the proliferative ability of limbal stem cells (Stapleton et al., 2017a, Ting and Ghosh, 2019, Notara et al., 2013).

4.4.5. Strengths and limitations

This study serves as one of the largest and most up-to-date examination of the risk factors, clinical characteristics and outcomes of BK in the UK. One of the limitations of this study was the inclusion of culture-negative BK cases. However, the medical case notes were examined to ensure that these cases were true BK cases based on the clinical presentation and the clinical course. In addition, inclusion of the culture-

negative cases enabled the examination of potential predictive factors for culture positivity and the outcome of these cases as culture-negative BK cases represents a large proportion of infectious keratitis in clinical practice. The issue with low culture yield in infectious keratitis has been consistently reported in many studies (Ting et al., 2021j, Ung et al., 2019b), highlight the need for improvement in the future.

In conclusion, BK represents a significant ocular morbidity in the UK. It not only significantly affects the patients' vision but also places considerable burden on the healthcare services as hospital admission is often required for intensive medical treatment and/or surgical intervention for BK. Affected patients are usually working adults (18-64 years) and hence the disease can have significant impact on the public and private workforce. As the visual outcome of BK is affected by the initial severity of the infection and the presenting vision, the importance of "prevention is better than cure" cannot be overemphasised. Ocular surface diseases, contact lens wear and systemic immunosuppression are important risk factors for BK and better preventative strategies need to be developed and targeted towards these areas. Future studies evaluating the risk factors and outcomes of other types of infectious keratitis, including fungal and Acanthamoeba keratitis, would be invaluable.

CHAPTER 5

Photoactivated Chromophore for Infectious Keratitis – Corneal Collagen Cross-Linking (PACK-CXL): A Systematic Review and Meta-Analysis

5.1. Introduction

Broad-spectrum antimicrobial therapy is currently the mainstay of treatment for IK; however, there is a decline in efficacy of antibiotic treatment due to an emerging trend of antimicrobial resistance in ocular infection (Asbell et al., 2015, Lalitha et al., 2017, Ung et al., 2019b). Furthermore, complications such as corneal melt, perforation and endophthalmitis, may ensue despite timely and intensive topical antibiotic treatment, necessitating further surgical interventions such as tectonic or therapeutic keratoplasty in a trial to preserve the eye and vision (Keay et al., 2006, Khor et al., 2018, Henry et al., 2012, Hossain et al., 2018, Ting et al., 2021a, Ting et al., 2021d). These issues highlight the need for alternative or adjuvant antimicrobial treatment to supplement the current therapeutic armamentarium for IK.

In the recent years, there has been an increasing interest in the use of therapeutic corneal cross-linking (PACK-CXL) for treating infectious keratitis. However, high-quality evidence is lacking in the literature, which results in a lack of adoption of such treatment in the clinical practice. To address the gap in the literature, this systematic review aimed to critically appraise the evidence and examine the efficacy and safety of PACK-CXL.

5.2. Methods

5.2.1. Protocol and registration

The systematic review title and protocol were registered with PROSPERO (registration number CRD42019131290) and the Joanna Briggs Institute Database of Systematic Reviews and Implementation Reports (Ting et al., 2020c).

5.2.2. Data sources and search methods

Two authors (DSJT and CH) searched MEDLINE (January 2003 to April 2019), EMBASE (January 2003 to April 2019), Cochrane Central Register of Controlled Trials (CENTRAL), ISRCTN registry (www.isrctn.com/), US National Institutes of Health Ongoing Trials Register ClinicalTrials.gov (http://clinicaltrials.gov) and World Health Organisation (WHO) International Clinical Trials Registry Platform (ICTRP) (www.who.int/ictrp) for primary research related to CXL for infectious keratitis or "PACK-CXL". The start date of January 2003 was selected because CXL was only introduced to clinical practice in 2003. There was no date restriction in the search for trials, however the search was restricted to English articles. Electronic databases were first searched on 05 May 2018, followed by a final update on 15 April 2019. Key words used were "cross-linking", "PACK-CXL", "riboflavin", "Vitamin B", "keratitis", "corneal ulcer", and "corneal infection". The bibliographies of included articles were independently and manually screened by two authors (DSJT and CH) to identify further relevant studies. Search strategies for MEDLINE and EMBASE are provided in the **Appendix 1**.

5.2.3. Study selection

All clinical studies, encompassing randomised controlled trials (RCTs), non-randomised controlled studies (NRS), case series and case reports, related to PACK-CXL were included as few RCTs were anticipated. The analysis was conducted at

two levels; (1) a meta-analysis of all eligible RCTs and (2) a systematic review of all clinical studies, including NRS, case series and case reports. All types of IK, including bacterial, fungal, viral, parasitic or mixed infection, were included in this review. Studies related to suspected non-infectious causes of keratitis or CXL used for non-antimicrobial purpose were excluded from this study. For the meta-analysis, the intervention group included cases of IK that were treated by PACK-CXL with standard antimicrobial therapy (SAT) whereas the control group included cases of IK that were treated with SAT alone. Restriction was made to publications in English but no restriction was applied to the location or setting of the study, or patients' demographic factors. This study conformed to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) guideline.

5.2.4. Data extraction

A web application designed for systematic reviews, Rayyan (Qatar), was used to help collate the potential studies and expedite the initial screening of abstracts and titles (Ouzzani et al., 2016). The titles and abstracts obtained from the searches were independently screened by two authors (DSJT and CH) to include studies that fulfilled the eligibility criteria. Both authors then independently assessed the full-text version of all the selected articles and extracted data onto a standardised data collection form for qualitative review. The extracted data included the authors and study title, year of publication, sample size, types of interventions, types of causative microorganisms, results and complications. Discrepancies were resolved by group consensus and independent adjudication (HSD) if consensus could not be reached.

For the meta-analysis, the following information were extracted from the included trials and entered into RevMan (Review Manager 5.3) software:

(1) Study characteristics: Year of publication, country of study, prospective registration of clinical trials, sample size, eligibility criteria, demographic factors, diagnostic criteria, method of randomisation, method of masking, number of study arms, number of participants, types of interventions, types of comparators, use of antimicrobial therapy in the intervention arm, source of funding, and any potential conflict of interest.

(2) Outcomes: Primary and secondary outcomes, risk of adverse events, complications during the procedure, post-procedure complications or secondary surgery, duration of follow-up, loss to follow-up and intervals at which outcomes were assessed.

5.2.5. Outcome measures

For the meta-analysis, the primary outcome measure was the time to complete corneal healing (defined as complete corneal re-epithelialisation and clearance of infiltrate and hypopyon; days) and the secondary outcome measures included the size of epithelial defect (mm^2) and size of infiltrate (mm^2) at 7 days and at final follow-up, visual acuity (LogMAR) at final follow-up, and risk of adverse events (defined as worsening IK and/or corneal melt or perforation requiring tectonic / therapeutic keratoplasty or evisceration) at final follow-up. A summary of the available data of all included studies was also performed and reported.

Continuous variables such as time to complete corneal healing, size of corneal epithelial defect and infiltrate, and corrected-distance-visual-acuity (CDVA) were presented as mean with standard deviation (SD). In studies that reported median and interquartile range, the means and SDs were estimated using formulas reported by Wan et al. (Wan et al., 2014) and the Cochrane Handbook estimator (Higgins and

Green, 2011b). Dichotomous variable such as risk of adverse events was defined by the number of participants with adverse events.

5.2.6. Assessment of risk of bias

Risk of bias was assessed by two authors (DSJT and CH) independently and any disagreement was adjudicated by HSD. Included RCTs were assessed for sources of systematic bias according to the guidelines in Chapter 8 of the Cochrane Handbook for Systematic Reviews of Interventions (Higgins and Green, 2011b). The review authors were not masked to the authors of the studies during this assessment. A judgement of 'high', 'low', or 'unclear' risk of bias was made for the following domains: (1) selection; (2) performance; (3) detection; (4) attrition; and (5) selective outcome reporting biases. NRS were assessed for risk of bias using the ROBINS-I tool (Sterne et al., 2016a) against seven domains; the worst judgement in any of the domains was used as the overall risk of bias.

5.2.7. Measure of treatment effect

Dichotomous data were measured as risk ratios (RRs) with 95% confidence intervals (CI) and continuous data as mean differences (MDs) with 95% CI. The unit of analysis was the participant and there was no issue with the unit of analysis in the included RCTs. The review was conducted based on the available data from the trials. When data were unavailable but the level of missing data and reasons for missing data in each group were similar, data were analysed even when intention-to-treat (ITT) analysis was not performed.

5.2.8. Assessment of heterogeneity

The heterogeneity of the RCTs and NRS was checked by careful review of the full-text, assessment of forest plots and examination of the I^2 value with its confidence

interval. The overall characteristics of the studies, in particular the types of participants and types of interventions were examined to assess the extent to which the studies were similar enough to make pooling study results sensible. The results of forest plots were reviewed for consistency of the size and direction of effects. I^2 values greater than 50% were considered indicative of substantial heterogeneity and meta-analysis could not be conducted due to inconsistency of effect estimates (Higgins and Green, 2011b). It was anticipated that some degree of heterogeneity will always exist due to clinical and methodological differences of the studies; therefore a random-effects model was used for the meta-analysis. The Chi² p-value was also considered as this has a low power when the number of studies were few. A p-value of <0.1 was considered statistically significant (Higgins and Green, 2011b).

5.2.9. Data synthesis and analysis

A meta-analysis was undertaken when there were sufficient similarities in the reporting of outcome measures. A random-effects model in RevMan 5.3 was used in view of the expected heterogeneity across different studies. The Mantel-Haenszel method was employed for analysing the risk ratio of adverse events in view of the small expected number of events. If there was inconsistency between the results of individual studies such that a pooled result might not be a good summary of the individual trial results – for example, the effects were in different directions or I^2 >50% and P <0.1 – the data were not pooled but described in narrative format. Where there was statistical heterogeneity, the data were pooled when all the effect estimates were in the same direction, such that a pooled estimate would seem to provide a good summary of the individual trial results. Sensitivity analysis was performed by assessing the impact of including studies at high risk of bias for an outcome in one or more key domains, when sufficient data were available. This was conducted by omitting each study in turn to examine the influence of individual studies on the overall

pooled estimate. A summary of findings is presented below including the assessment of the quality of the evidence for outcomes using the GRADE approach with GRADE Pro/GDT software (Schunemann et al.). All RCTs were started with a rating of 'high-quality' evidence and were downgraded by one level for serious concerns (or by two levels for very serious concerns) regarding the risk of bias, inconsistency, indirectness, imprecision or publication bias. The quality of evidence of studies was graded by two assessors (DSJT and CH) independently and any disagreement was adjudicated by HSD.

5.2.10. Assessment of adverse events

The risk of adverse events was graphically represented on albatross plots generated by the module installed on STATA 15.1 statistical software (Harrison et al., 2017). Pooled estimates of the risk of adverse events across comparative studies, including RCTs and NRS, were calculated. The risk of adverse events of PACK-CXL was summarised according to the type of IK. Studies were categorised as bacterial, fungal, Acanthamoeba, viral, mixed or culture-negative presumed IK cohort. Mixed IK cohort referred to studies that included more than one group of causative microorganisms.

5.3. Results

5.3.1. Literature search and study characteristics

The electronic searches last conducted on 15 April 2019 retrieved a total of 754 titles and abstracts (see **Figure 5.1** for the PRISMA flow chart). After removing 181 duplicates and including two additional records identified through other sources, the remaining 573 records were screened and 421 references that were not relevant to the scope of the review were excluded. A total of 52 full-text copies of papers were assessed for eligibility. After excluding 6 ineligible articles (Ammermann et al., 2014,

Hager et al., 2016, Idrus et al., 2018, Mattila et al., 2013, Schnitzler et al., 2000, Wu et al., 2013a), 46 studies were included in the systematic review. These included four RCTs (Bamdad et al., 2015, Kasetsuwan et al., 2016, Said et al., 2014, Uddaraju et al., 2015), two NRS (Basaiawmoit et al., 2018, Vajpayee et al., 2015), 20 case series (Agrawal and Singh, 2016, Chan et al., 2014, Cristian et al., 2019, Erdem et al., 2018, Iseli et al., 2008, Khalili et al., 2017, Khan et al., 2011, Knyazer et al., 2018, Li et al., 2013b, Makdoumi et al., 2010, Makdoumi et al., 2012, Muller et al., 2012, Panda et al., 2012, Price et al., 2012, Ramona et al., 2016, Rosetta et al., 2013, Shetty et al., 2014, Skaat et al., 2014, Sorkhabi et al., 2013, Zloto et al., 2018), and 20 case reports (Abbouda et al., 2014, Anwar et al., 2011, Arance-Gil et al., Casagrande et al., 2014, Chan et al., 2017, Demirci and Ozdamar, 2013, Ferrari et al., 2013, Ferrari et al., 2009, Garduno-Vieyra et al., 2011, Igal et al., 2017, Kozobolis et al., 2010, Kymionis et al., 2016, Labiris et al., 2014, Moren et al., 2010, Oflaz et al., 2017, Passilongo et al., Saglk et al., 2013, Tabibian et al., 2014, Yagci et al., 2016, Zarei-Ghanavati and Irandoost, 2015), examining the efficacy and safety of PACK-CXL in 435 participants (438 eyes) with IK, of which 311 participants received PACK-CXL with SAT, 15 participants received PACK-CXL alone, and 112 participants received SAT alone. All studies included one eye per participant except for Chan et al. study (Chan et al., 2014) and Cristian et al. study (Cristian et al., 2019). Within the group that received PACK-CXL with/without SAT, the causative microorganisms included 152 (46.6%) bacteria, 89 (27.3%) fungi, 20 (6.1%) Acanthamoeba, 4 (1.2%) viruses, 20 (6.1%) mixed, and 41 (12.6%) culture-negative presumed IK. The main characteristics of all RCTs, including the authors' name, year of publication, number of treated participants, treatment protocol used, types of causative microorganisms, severity of IK, main results, adverse events, and visual outcome (if available), are summarised in **Appendix 2 3**. Outcomes of RCTs are analysed and summarised under the meta-analysis section. In addition, two ongoing RCTs were identified from the searches of clinical trial registries

(https://clinicaltrials.gov/ct2/show/NCT02570321 and

https://clinicaltrials.gov/ct2/show/NCT02717871).

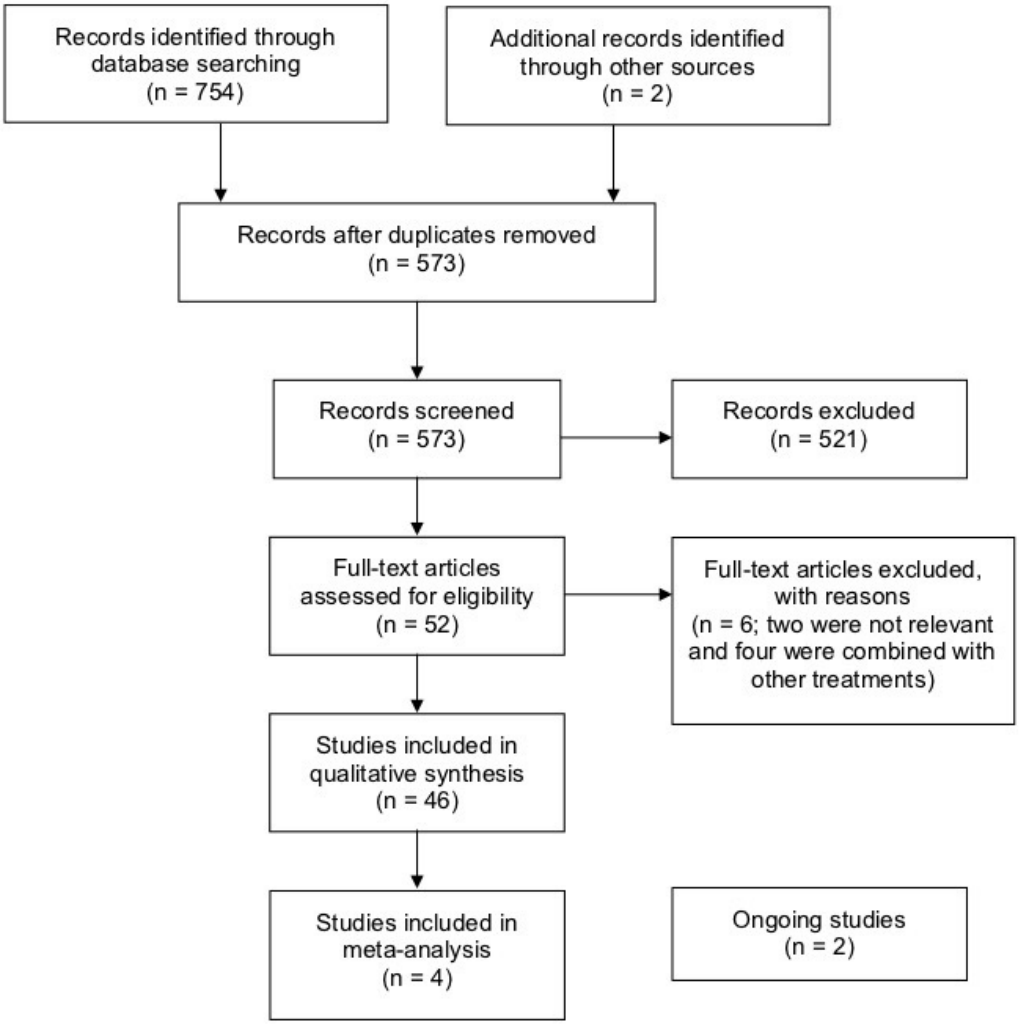


Figure 5.1. PRISMA flow diagram of the literature search for PACK-CXL.

5.3.2. Meta-analysis of eligible RCTs

5.3.2.1. Overall description

The four RCTs included a total of 115 participants, with 58 participants receiving PACK-CXL plus SAT (intervention group) and 57 participants receiving SAT alone (control group). These consisted of four single-centered RCTs, which was conducted separately in Iran (Bamdad et al., 2015), Thailand (Kasetsuwan et al., 2016), Egypt (Said et al., 2014), and India (Uddaraju et al., 2015). Trials included participants aged between 15 and 84 years (mean age of 40 – 56 years), with slight male preponderance (59.1%). Two RCTs (Kasetsuwan et al., 2016, Uddaraju et al., 2015) were prospectively registered with the clinicaltrials.gov (NCT01831206 and NCT02328053).

5.3.2.2. Types of microbes and severity of IK

The RCTs were heterogeneous in terms of the types of infection. Bamdad et al. (2015) and Uddaraju et al. (2015) included bacterial keratitis alone and fungal keratitis alone, respectively, whereas Kasetsuwan et al. (2016) included both bacterial and fungal keratitis in their studies. Said et al. (2014) included bacterial, fungal, Acanthamoeba and mixed infection. None of the studies included viral infection. The collective microbiological profiles included 53 (46.1%) bacteria, 30 (26.1%) fungi, 3 (2.6%) Acanthamoeba, 8 (7.0%) mixed bacteria/fungi, and 21 (18.3%) culture-negative presumed IK. The proportion of the types of organisms was similar between intervention and control arms in all RCTs, except for one RCT (Said et al., 2014) where there was a significantly higher proportion of mixed bacteria/fungi infection in the intervention arm (7, 33.3%) compared to the control arm (1, 5.3%; p=0.027).

5.3.2.3. Treatment protocols and outcome measures

All four trials compared PACK-CXL plus SAT with SAT alone. Dresden CXL protocol, using an irradiance of UVA of 3 mW/cm^2 for 30 mins (total fluence 5.4mJ/cm^2), was employed in all intervention arms. After enrolment the participants were treated with PACK-CXL immediately on the first day of presentation in two studies (Bamdad et al., 2015, Kasetsuwan et al., 2016), within 48 hours in one study (Said et al., 2014), and after two weeks of non-improvement with SAT in one study (Uddaraju et al., 2015). The primary and secondary outcome measures used in these RCTs are summarised in **Table 5.1**. They included size of stromal infiltrate and epithelial defect at 7 days and final follow-up (14 days or 30 days) (Bamdad et al., 2015, Kasetsuwan et al., 2016), time to complete corneal healing or treatment duration (defined as complete re-epithelialisation of the ulcer and disappearance of the infiltrate and hypopyon) (Said et al., 2014, Bamdad et al., 2015), adverse events (Said et al., 2014) (Uddaraju et al., 2015) (defined by corneal perforation and/or endophthalmitis and/or increase in infiltrate size by ≥2mm), and visual-acuity (Kasetsuwan et al., 2016, Said et al., 2014, Uddaraju et al., 2015). The follow-up duration was between four and six weeks' post-treatment, except for one study which the duration was not specified (Said et al., 2014). The lack of similarity in outcome measures limited the possibility for combining all data from individual RCTs.

Table 5.1. Outcome measures used in randomised controlled trials of PACK-CXL.

Authors (year)	Primary Outcome Measures	Secondary Outcome Measures
Kasetsuwan et al. (2016)	(1) Size of stromal infiltrate	(1) Size of epithelial defect (2) Treatment failure (3) BPVA
Uddaraju et al. (2015)	(1) Treatment failure at 6 weeks	(1) UDVA
Bamdad et al. (2015)	Not specified but following parameters were analysed and reported: (1) Size of epithelial defect (2) Size of stromal infiltrate (3) Grade of corneal ulcer (4) Duration of treatment (5) Treatment failure	
Said et al. (2014)	Not specified but following parameters were analysed and reported: (1) Time to complete healing (defined by complete corneal epithelialisation and clearance of infiltrate) (2) CDVA (3) Treatment failure	

BPVA = Best-corrected-pinhole-visual-acuity; UDVA = Uncorrected distance visual acuity; CDVA = Corrected distance visual acuity

5.3.2.4. Risk of bias

Risk of bias was for RCTs determined by using the “risk of bias” assessment tool. Sequence generation, allocation concealment, masking of participants, personnel and outcome assessors, incomplete outcome data, selective outcome reporting and potential threats to validity were considered (**Figure 5.2**). Sequence generation was unclear in two of the studies (Bamdad et al., 2015, Kasetsuwan et al., 2016) where simple randomisation was performed but the method was not clearly stated, potentially increasing the risk of bias. No details of attempts to conceal allocation of intervention assignment were given in two trials (Bamdad et al., 2015, Said et al., 2014). Masking of participants was not possible but masking of assessors was possible in most studies. All trials had complete data except Uddaraju et al. (2015) which reported outcomes for 13 participants of the intended 31 participants with fungal keratitis, as the trial was terminated early due to the concern of corneal perforation rate in the PACK-CXL group. Selective reporting was not considered to be a problem in the included trials but it was not always possible to assess this risk of bias adequately for one trial (Said et al., 2014).

Risk of bias for NRS was determined by using the ROBINS-I grading criteria. Two NRS identified from database search were graded as having critical risk of bias (score 4), as both studies showed selection bias of participants and lacked balance of unknown confounding factors (Basaiawmoit et al., 2018, Vajpayee et al., 2015). Both studies had post-intervention bias in terms of the care provided and measurement of outcomes. One study had bias due to missing follow-up data (Basaiawmoit et al., 2018).

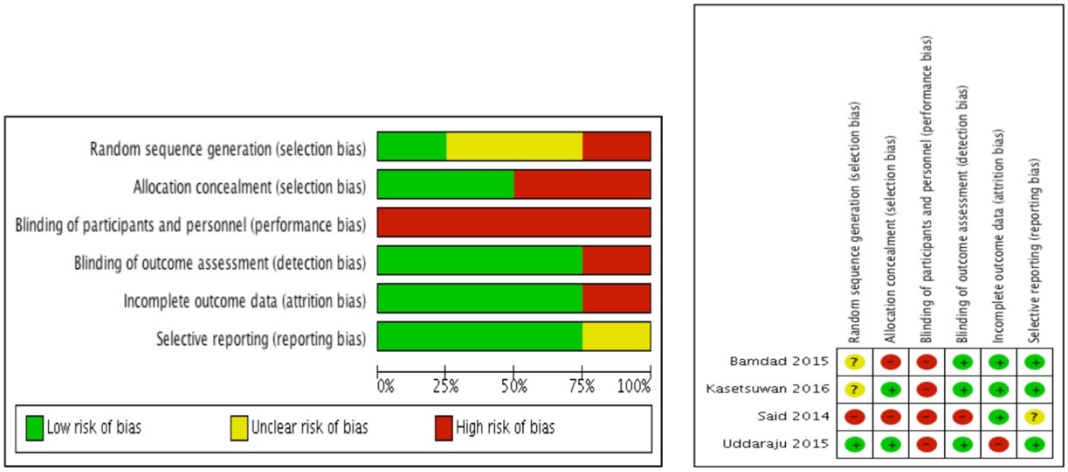


Figure 5.2. Risk of bias graph of RCTs on PACK-CXL.

Review authors' judgements about each risk of bias item presented as percentages across all included studies.

Table 5.2. GRADE summary of findings of PACK-CXL compared standard antimicrobial treatment for infectious keratitis.

Patient or population: Infectious Keratitis; **Intervention**: PACK-CXL; **Comparison**: Standard antimicrobial treatment

Outcomes	№ of participants (studies)	Certainty of the evidence (GRADE)	Relative effect (95% CI)	Anticipated absolute effects	
				Risk with standard antimicrobial treatment	Risk difference with PACK-CXL*
Mean time to healing	72 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b]	-	24.7 to 46.1 days	MD 7.44 days shorter (10.71 shorter to 4.16 shorter)
Size of epithelial defect at 7 days	62 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b]	-	14.94 to 20.9 mm^2	MD 3.66 mm^2 smaller (14.26 smaller to 6.94 smaller)
Size of epithelial defect at final follow-up (14 to 30 days)	62 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b,c,d]	-	4.9 to 8.31 mm^2	MD 5.09 mm^2 smaller (9.43 smaller to 0.74 smaller)
Size of Infiltrates at 7 days	62 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b]	-	14.88 to 28.1 mm^2	MD 5.49 mm^2 smaller (7.44 smaller to 3.54 smaller)
Size of infiltrate at final follow-up (14 to 30 days)	62 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b,d]	-	9.3 to 9.63 mm^2	MD 5.27 mm^2 smaller (9.12 smaller to 1.41 smaller)
CDVA (LogMAR) at final follow-up (one to three months)	70 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b,d]	-	1.2 to 1.67	MD 0.08 LogMar worse (0.21 better to 0.37 worse)
Adverse events at final follow-up (one to three months)	102 (3 RCTs)	⊕◯◯◯ VERY LOW [a,b,d,e]	RR 0.49 (0.11 to 2.29)	140 per 1,000	71 fewer per 1,000 (125 fewer to 181 more)

*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

CI: Confidence interval; **MD:** Mean difference; **RR:** Risk ratio

Explanations

a. High risk of bias due to lack of allocation concealment and blinding.
b. Total number of participants is less than the number generated by a conventional sample size calculation.
c. There is significant heterogeneity.
d. Differences in final follow-up
e. There are few events and the confidence interval includes appreciable benefit and harm.

5.3.2.5. Effects of interventions

The effects of interventions were categorised into: (A) time to complete corneal healing; (B) size of epithelial defect; (C) size of infiltrate; (D) corrected-distance-visual-acuity (CDVA) in LogMAR; and (E) risk of adverse events. The GRADE summary of findings for each treatment outcome is summarised in **Table 5.2.**

5.3.2.5.1. Time to complete corneal healing

Two RCTs reported the time to complete corneal healing (or treatment duration), which was defined as complete re-epithelialisation and disappearance of infiltrate and hypopyon (Bamdad et al., 2015, Said et al., 2014). There is very low-quality evidence that adjuvant PACK-CXL shortened the time to complete healing compared to SAT alone (MD -7.44 days; 95% CI -10.71 to -4.16; I^2=0%; p<0.0001) (**Figure 5.3A**). Notably, the size of corneal ulcer in the adjuvant PACK-CXL group was significantly larger than the control group at baseline in one study (Said et al., 2014). Quality of evidence was downgraded due risk of bias and imprecision. There was a lack of allocation concealment and blinding. The total number of participants pooled in the meta-analysis were less than the number generated by a conventional sample size calculation.

5.3.2.5.2. Size of epithelial defect

Two studies reported the size of epithelial defect at 7 days and final follow-up (Kasetsuwan et al., 2016, Bamdad et al., 2015). There is very low-quality evidence that adjuvant PACK-CXL was equally effective as SAT alone in reducing the size of corneal epithelial defect at 7 days follow up (MD -3.66 mm^2; 95% CI -14.26 to 6.94; I^2=50%; p=0.50) (**Figure 5.3B**). The quality of evidence was downgraded due to risk of bias, inconsistency and imprecision. In terms of the size of epithelial defect at final follow-up, there was heterogeneity between the two studies so meta-analysis was not

performed (I^2=58%). There is very low-quality evidence that adjuvant PACK-CXL was equally effective as SAT alone in reducing the size of corneal epithelial defect at final follow up. The quality of evidence was downgraded due to risk of bias, inconsistency, indirectness and imprecision. Final follow-up differed between 14 days and 30 days. The observed heterogeneity could be due to the mixed cohort of bacterial and fungal keratitis cases included in Kasetsuwan et al. study (Kasetsuwan et al., 2016) where 60% of the cases were fungal. When analysis of only bacteria keratitis cases was performed, the size of epithelial defect at final follow-up favored PACK-CXL (MD -6.60; 95% CI -9.64 to -3.57; p<0.0001) (Bamdad et al., 2015, Kasetsuwan et al., 2016).

5.3.2.5.3. Size of infiltrate

Two studies reported the size of infiltrate at 7 days and final follow-up (14 days or 30 days) (Bamdad et al., 2015, Kasetsuwan et al., 2016). There is very low-quality evidence that adjuvant PACK-CXL was more effective than SAT alone at reducing the size of infiltrate at 7 days' (MD -5.49mm^2; 95% CI -7.44 to -3.54; I^2=0%; p<0.0001) and at final follow-up (MD -5.27mm^2; 95% CI -9.12 to -1.41; I^2=21%; p=0.007) (**Figure 5.3C**). The quality of the evidence was downgraded due to high risk of bias and imprecision.

5.3.2.5.4. Visual acuity

There is very low-quality evidence that there was no difference in the mean CDVA at the final follow-up between the adjuvant PACK-CXL group and the SAT group (0.08; 95% CI -0.21 to 0.37; I^2=9%; two RCTs with 70 participants) (Kasetsuwan et al., 2016, Said et al., 2014) (**Figure 5.3D**). The quality of the evidence was downgraded due to high risk of bias and imprecision.

5.3.2.5.5. Adverse events

Adverse events were defined as worsening IK and/or corneal melt / perforation requiring tectonic / therapeutic keratoplasty or evisceration. Bamdad et al. (2015) reported no event of corneal perforation in both treatment arms though three participants required secondary surgeries such as amniotic membrane transplant (AMT) or conjunctival flap (one in PACK-CXL group and two in standard care group). For the other three RCTs, a total of eight participants randomised to PACK-CXL required therapeutic keratoplasty or evisceration for uncontrolled IK compared to 11 participants that received SAT (Kasetsuwan et al., 2016, Said et al., 2014, Uddaraju et al., 2015). There is very low-quality evidence that adjuvant PACK-CXL patients did not reduce the rate of adverse events when compared to SAT alone [Risk ratio (RR) 0.84; 95% CI 0.26 to 2.71; p=0.77; four studies with 115 participants]. A sensitivity analysis was performed with the exclusion of Uddaraju et al. study because early trial termination might bias the effect estimate (Uddaraju et al., 2015). When excluded, there was no statistically significant change in the effect estimate on the risk of adverse events between adjuvant PACK-CXL and SAT alone (RR 0.49; 95% CI 0.11 to 2.29; I^2=22%; p=0.37; three RCTs with 102 participants) (**Figure 5.3E**). The quality of evidence was downgraded due to high risk of bias and imprecision. There are few events and the confidence interval included appreciable benefit and harm. Two studies did not report the type of microorganism involved in cases that were complicated by corneal perforation (Kasetsuwan et al., 2016, Said et al., 2014). According to consort harms reporting guidance, the quality of harms reporting was considered inadequate in three included studies where the severity of adverse events and clinical sequalae were not clearly described (Kasetsuwan et al., 2016, Said et al., 2014, Uddaraju et al., 2015).

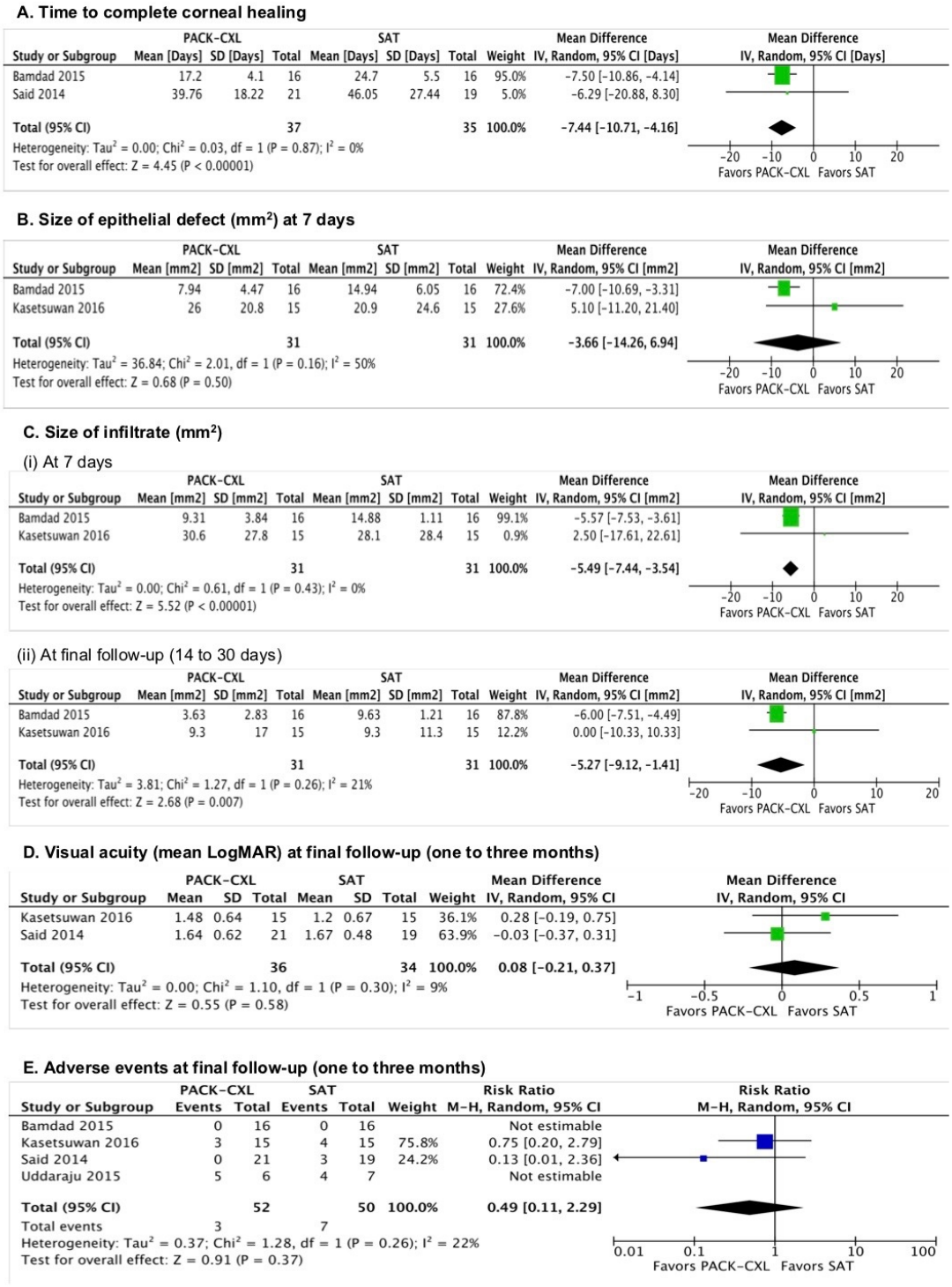


Figure 5.3. Forest plots of various outcomes of PACK-CXL.

Summary of the meta-analysis (forest plot) comparing the efficacy between PACK-CXL plus standard antimicrobial treatment (SAT) and SAT alone in eligible randomized controlled trials, in terms of: **(A)** time to complete corneal healing; **(B)** size of epithelial defect; **(C)** size of stromal infiltrate; **(D)** corrected-distance-visual-acuity; and **(E)** risk of adverse events.

5.3.2.5.6. Subgroup analysis

There were insufficient RCTs to perform subgroup analysis between bacterial and fungal keratitis outcomes.

As effectiveness RCTs are lacking, NRS were included in the assessment of time to complete healing and adverse events. The mean time to complete corneal healing and number of adverse events reported in all comparative interventional studies, including RCTs and NRS, are summarised in albatross plots (**Figure 5.4**). All comparative studies investigating time to complete healing favoured adjuvant PACK-CXL (Bamdad et al., 2015, Basaiawmoit et al., 2018, Said et al., 2014, Vajpayee et al., 2015), with one study of bacterial keratitis showed statistical significance (Bamdad et al., 2015). In terms of the risk of adverse events, one study showed negative association favouring adjuvant PACK-CXL in mixed IK cohort and one study showed a positive association favoring SAT alone in fungal keratitis cohort; however none of the comparative studies showed statistical significance (Basaiawmoit et al., 2018, Kasetsuwan et al., 2016, Said et al., 2014, Uddaraju et al., 2015, Vajpayee et al., 2015). Pooled risk estimates of adjuvant PACK-CXL on the risk of adverse events in comparative studies were grouped and analysed according to type of IK cohorts (**Table 5.3**). It was found that adjuvant PACK-CXL did not significantly influence the risk of adverse events in mixed IK (RR 0.57; 95% CI 0.20 to 1.61) and fungal keratitis cohorts (RR 1.30; 95% CI 0.66 to 2.54).

Case series and case reports were reviewed for uncommon or unexpected adverse events. Analysis of the outcomes of the PACK-CXL, based on different types of causative microorganisms, are summarised in **Table 5.4**. Studies that reported pooled results with no distinction among the causative microorganisms were excluded from the subgroup analysis. Based on large NRS (≥ 5 participants for a particular type of microorganism) (Khalili et al., 2017, Knyazer et al., 2018, Makdoumi

et al., 2012, Cristian et al., 2019, Price et al., 2012, Ramona et al., 2016, Shetty et al., 2014, Sorkhabi et al., 2013, Zloto et al., 2018, Erdem et al., 2018, Li et al., 2013b, Vajpayee et al., 2015, Agrawal and Singh, 2016, Panda et al., 2012), the overall rate of complete corneal epithelial healing was 84.8% (78/92) for bacterial keratitis, 73.5% (36/49) fungal keratitis, 66.7% (4/6) Acanthamoeba keratitis, and 92.0% (23/25) culture-negative presumed IK. There was insufficient evidence on the use of PACK-CXL in Acanthamoeba and mixed infection whereas viral keratitis was considered a contraindication for PACK-CXL in the majority of the studies. Based on small case series and case reports, the complete healing rate was reported to be 75% (9/12) for Acanthamoeba keratitis, 33% (1/3) for mixed bacterial / fungal keratitis, 75% (3/4) for mixed Acanthamoeba / fungal keratitis, 100% (1/1) for mixed bacterial / Acanthamoeba keratitis, and 25% (1/4) for viral keratitis.

5.3.3. Characteristics of ongoing studies

Two ongoing RCTs, comparing PACK-CXL and/or SAT with SAT alone, were identified from the searches of clinical trial registries. One RCT, named Cross-linking for Corneal Ulcers Treatment Trial (CLAIR), is recruiting 266 participants with bacterial or fungal keratitis (https://clinicaltrials.gov/ct2/show/NCT02570321). The primary outcome measure is the microbiological cure on repeat culture after adjuvant PACK-CXL or SAT alone. The other RCT, named Swiss PACK-CXL Multicenter Trial for the Treatment of Infectious Keratitis, is enrolling 252 participants with bacterial, fungal or mixed keratitis (https://clinicaltrials.gov/ct2/show/NCT02717871). This trial is investigating PACK-CXL as a primary treatment alone in early IK. The time to re-epithelialisation of the cornea and time from treatment to discharge of patient are set as the primary and secondary outcome measures, respectively.

Figure 5.4. Albatross plot for assessing mean time to healing after PACK-CXL.

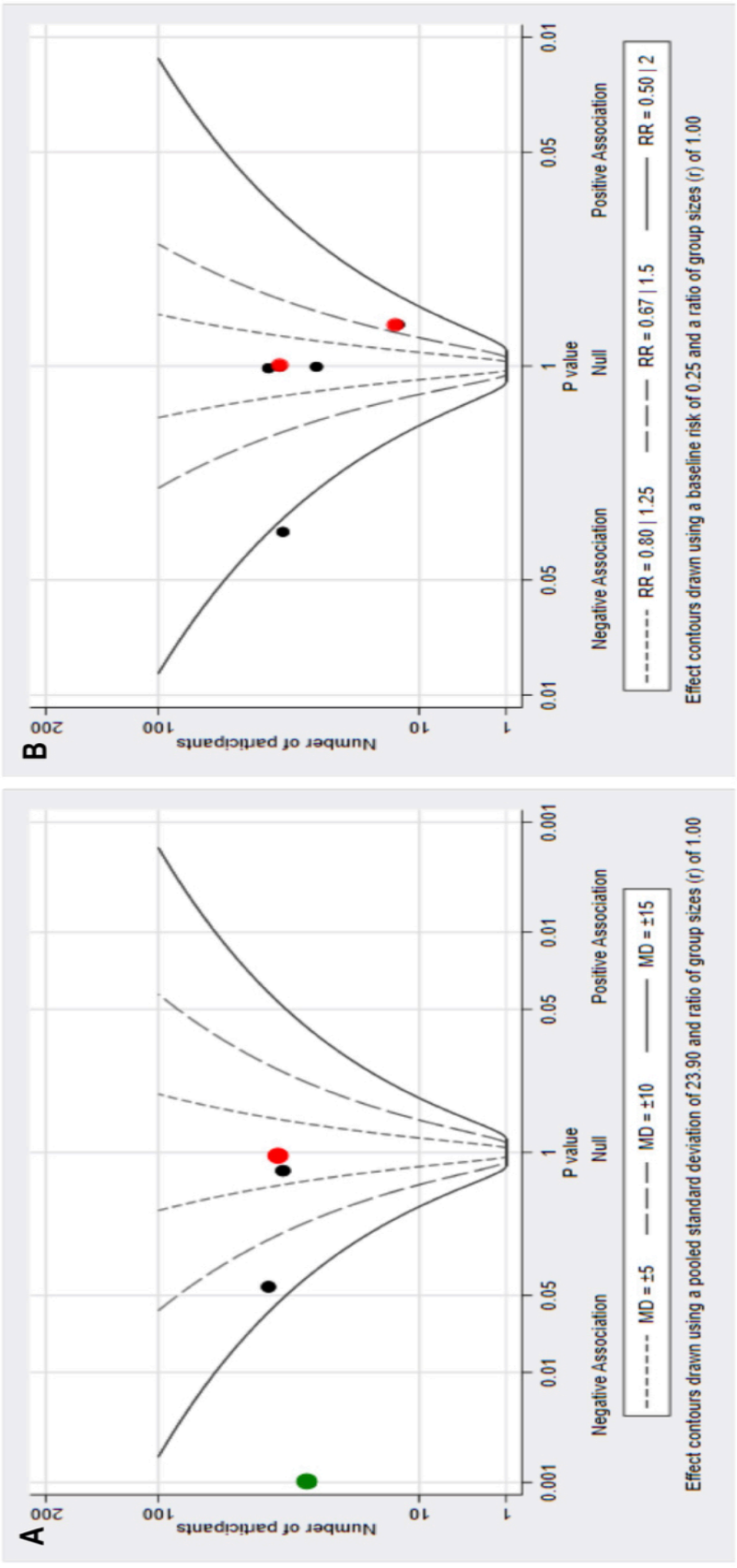


Figure 5.4. **(A)** Albatross plot for studies comparing the mean time to healing of adjuvant PACK-CXL with standard antimicrobial treatment (SAT) alone, with contours for mean differences (MDs). Red points represent comparative studies of fungal keratitis, black points represent mixed infectious keratitis cohorts, and green point represents study that included bacterial keratitis. Negative association favors the use of adjuvant PACK-CXL and positive association favors the use of SAT alone. Points lying outside the p-value of 0.05 were considered statistically significant. **(B)** Albatross plot for studies comparing adverse events of adjuvant PACK-CXL with SAT alone for infectious keratitis with contours for relative risk (RR). Red points represent comparative studies of fungal keratitis and black points represent mixed infectious keratitis cohorts. Negative association favors the use of adjuvant PACK-CXL and positive association favors the use of SAT alone. Points lying outside the p-value of 0.05 were considered statistically significant.

Table 5.3. Pooled risk estimates of PACK-CXL on risk of adverse events.

IK cohorts	Number of Studies	Types of studies	Number of participants	Estimate of magnitude (95% CI)
Bacterial	1	1 RCT	32	NK
Mixed*	4	2 RCTs 1 NRS	115	RR 0.57 (0.20 to 1.61)
Fungal	2	1 RCT 1NRS	54	RR 1.30 (0.66 to 2.54)

RCT = Randomised controlled trial; NRS = Non-randomised controlled studies; CI = Confidence interval; RR = Risk ratio; NK = Not known

*Mixed IK cohorts refer to cohorts consisting of two or more types of IK, which could be either bacterial, fungal, Acanthamoeba or mixed IK.

Table 5.4. Summary of the healing rate and treatment failure of PACK-CXL in non-RCT studies.

Authors	Year	Numbers	Complete healing	Healing time (days)	Treatment failure
Bacterial keratitis					
Agrawal et al.	2016	1	1 (100%)	-	0 (0%)
Anwar et al.	2011	1	1 (100%)	14	0 (0%)
Casagrande et al.	2014	1	1 (100%)	60	0 (0%)
Chan et al.	2014	2	1 (50%)	90	0 (0%)
Chan et al.	2017	1	1 (100%)	14	0 (0%)
Ferrari et al.	2009	1	1 (100%)	30	0 (0%)
Iseli et al.	2008	3	3 (100%)	28 – 120	0 (0%)
Khalili et al.	2017	6	4 (67%)	-	2 (33%)
Knyazer et al.	2018	9	9 (100%)	4 – 30	0 (0%)
Kozobolis et al.	2010	1	1 (100%)	7	0 (0%)
Labiris et al.	2014	1	1 (100%)	5	0 (0%)
Makdoumi et al.	2012	12	12 (100%)	1 – 14	0 (0%)
Makdoumi et al.	2010	4	4 (100%)	-	0 (0%)
Muller et al.	2012	3	2 (67%)	120	0 (0%)
Oflaz et al.	2017	1	1 (100%)	30	1 (33%)
Price et al.	2012	24	20 (83%)	4 – 40	0 (0%)
Ramona et al.	2016	10	8 (80%)	30	3 (17%)*
Rosetta et al.	2013	3	3 (100%)	3 – 45	2 (20%)
Shetty et al.	2014	9	6 (67%)	14 – 28	0 (0%)
Skaat et al.	2014	3	2 (67%)	-	3 (33%)
Sorkhabi et al.	2013	9	7 (78%)	-	1 (33%)
Zloto et al.	2018	13	12 (92%)	-	2 (22%)
					1 (8%)
Fungal keratitis					
Abbouda et al.	2014	2	0 (0%)	-	1 (50%)
Anwar et al.	2011	1	1 (100%)	60	0 (0%)
Erdem et al.	2018	9	4 (44%)	15 – 33	4 (44%)
Igal et al.	2017	1	1 (100%)	-	0 (0%)
Iseli et al.	2008	2	1 (50%)	-	1 (50%)
Knyazer et al.	2018	1	1 (100%)	16	0 (0%)
Li et al.	2013	8	8 (100%)	3 – 8	0 (0%)
Muller et al.	2012	2	1 (50%)	120	1 (50%)
Price et al.	2012	6	3 (50%)	26 – 28	3 (50%)
Saglk et al.	2013	1	1 (100%)	7 (after 2nd CXL)	0 (0%)
Shetty et al.	2014	6	3 (50%)	15 – 90	3 (50%)
Sorkhabi et al.	2013	1	1 (100%)	-	0 (0%)
Tabibian et al.	2014	1	1 (100%)	3	0 (0%)
Vajpayee et al.	2015	20	18 (90%)	15 – 90	2 (10%)
Yagci et al.	2016	1	0 (0%)	-	0 (0%)

Acanthamoeba keratitis					
Arance-Gil et al.	2014	1	0 (0%)	-	1 (100%)
Chan et al.	2014	1	1 (100%)	60	0 (0%)
Cristian et al.	2019	6	4 (67%)	59 – 217	0 (0%)
Demirci et al.	2013	1	1 (100%)	10	0 (0%)
Garduno-Vieyra et al.	2011	1	1 (100%)	21	0 (0%)
Khan et al.	2011	3	3 (100%)	21 – 42 31	0 (0%)
Moren et al.	2010	1	1 (100%)	-	0 (0%)
Muller et al.	2012	1	1 (100%)	54 – 145	0 (0%)
Price et al.	2012	2	0 (0%)	10	
Rosetta et al.	2013	1	1 (100%)		0 (0%)
Viral keratitis					
Ferrari et al.	2013	1	0 (0%)	-	1 (100%)
Khalili et al.	2017	2	1 (50%)	25	1 (50%)
Price et al.	2012	1	0 (0%)	-	0 (0%)
Mixed bacterial / fungal keratitis					
Erdem et al.	2018	2	1 (50%)	-	1 (50%)
Knyazer et al.	2018	1	0 (0%)	-	1 (100%)
Mixed acanthamoeba / fungal keratitis					
Erdem et al.	2018	2	2 (100%)	15 – 35	0 (0%)
Kymionis et al.	2016	1	1 (100%)	60	0 (0%)
Passilongo et al.	2018	1	0 (0%)	-	1 (100%)
Mixed acanthamoeba / bacterial keratitis					
Panda et al.	2012	1	1 (100%)	8	0 (0%)
Culture negative					
Agrawal et al.	2016	5	3 (60%)	-	2 (40%)
Chan et al.	2014	1	1 (100%)	120	0 (0%)
Knyazer et al.	2018	9	9 (100%)	-	0 (0%)
Kozobolis et al.	2010	1	1 (100%)	7	0 (0%)
Makdoumi et al.	2012	4	4 (100%)	4 – 12	0 (0%)
Makdoumi et al.	2010	3	3 (100%)	-	0 (0%)
Panda et al.	2012	6	6 (100%)	5 – 18	0 (0%)
Skaat et al.	2014	3	3 (100%)	19 – 47	0 (0%)
Zarei-Ghanavati et al.	2015	1	1 (100%)	14 -	0 (0%)
Zloto et al.	2018	5	5 (100%)		0 (0%)

*One patient lost to follow-up.

PACK-CXL = Photoactivated chromophore for infectious keratitis-corneal collagen cross-linking; AMT = Amniotic membrane transplant

5.4. Discussion

This study represents the most up-to-date and comprehensive systematic review and meta-analysis examining the effectiveness and safety of PACK-CXL in 435 participants (438 eyes), of which 325 participants received PACK-CXL with / without SAT. Since the last systematic review on PACK-CXL conducted by Abbouda et al. (2018) (consisting of 21 studies of 145 eyes) and Papaioannou et al. (2016) (consisting of 25 studies of 210 eyes) in 2016, a further 21 studies (including two additional RCTs) of 215 participants have been published. The doubling amount of literature on PACK-CXL in the recent years highlights a growing demand for innovative antimicrobial treatment for refractory IK, and the increasingly challenge faced by the clinicians in managing advanced and complex cases of IK. Both previous systematic reviews highlighted the potential utility of PACK-CXL but further high-quality RCTs were required (Abbouda et al., 2018, Papaioannou et al., 2016). However, the conclusion was not based on the findings of meta-analysis, primarily due to the limited number of published RCTs during the conduct of the systematic reviews.

5.4.1. Summary of main findings

In this systematic review, four RCTs with 58 participants in the intervention group (PACK-CXL with SAT) and 57 participants in the control group (SAT alone) were included. The majority of the studies focused on either bacterial or fungal keratitis or a combination of both, with only one RCT including Acanthamoeba keratitis (three participants). None of the RCTs examined the utility of PACK-CXL in viral keratitis. Based on the meta-analysis of two RCTs with 72 participants, adjuvant PACK-CXL significantly shortened the time to complete corneal healing by 7 days when compared to SAT alone. One study (Bamdad et al., 2015) included only participants with bacterial keratitis and the other study primarily included bacterial, fungal and

mixed IK (Said et al., 2014). In addition adjuvant PACK-CXL was superior to SAT alone in terms of the resolution of the size of infiltrate at 7 days and at final follow-up (14-30 days) (Bamdad et al., 2015, Kasetsuwan et al., 2016). These two RCTs examined a total of 62 participants with 44 culture-positive and presumed bacterial keratitis and 18 culture-positive and presumed fungal keratitis. Both studies included primarily moderate IK (2-6 mm ulcer size and involved up to the anterior two third of the stroma) and severe IK (>6 mm ulcer size and involved the posterior one third of the stroma).

In terms of other outcomes, adjuvant PACK-CXL did not reduce the risk of adverse events such as corneal perforation when compared to SAT alone group. While three RCTs showed a similar or lower risk of adverse events in the PACK-CXL group, Uddaraju et al. (Uddaraju et al., 2015) reported an opposite trend in their study. The latter study included cases of severe and refractory fungal keratitis that involved the posterior two third of the cornea and did not respond to the standard anti-fungal treatment for at least two weeks. Their findings might be confounded by the difference in the baseline severity of the fungal keratitis where the PACK-CXL group was considerably worse than the SAT group. Based on their findings, they have cautioned the use of PACK-CXL in patients with severe deep fungal infection. When Uddaraju et al. study was excluded from the analysis (due to early termination and significant difference in the baseline severity of the ulcer), there was an increase in direction favoring towards the use of adjuvant PACK-CXL in reducing the risk of adverse events (RR 0.49) but the difference was not statistically significant. There was also no significant difference between adjuvant PACK-CXL and SAT alone for other outcomes such as the size of epithelial defect and CDVA.

Based on NRS and case series (≥ 5 participants for a particular type of microorganism), a complete healing rate of 84.8% (78/92) for bacterial keratitis,

73.5% (36/49) fungal keratitis, 66.7% (4/6) Acanthamoeba keratitis, and 92.0% (23/25) culture-negative presumed IK was observed. Only one participant (1.1%) in the bacterial keratitis group and three participants (6.1%) in the fungal keratitis group required additional AMT to help achieve complete re-epithelialisation. These findings suggest that PACK-CXL may serve as a useful adjuvant therapy for bacterial and fungal keratitis. However, there were insufficient data to support the adjuvant use of PACK-CXL in Acanthamoeba, mixed and viral IK.

5.4.2. Overall completeness and applicability of evidence

Overall the evidence was very low-quality with only four RCTs reporting small sample sizes. Price et al. (2012) and Hafezi and Kling (2016) had previously performed sample size power calculation for evaluating the effectiveness of PACK-CXL and had highlighted that around 200-250 participants were required. However, the sample size in all four included RCTs was substantially underpowered in efficacy and safety outcomes. Small sample sizes precluded any subgroup analysis of different causative microorganisms.

The other limiting issue with the meta-analysis was the inconsistency in measurement and reporting of the treatment outcomes among the four included RCTs. There was a total of eight different outcomes being reported and risk of adverse events was the only outcome that was consistently examined in all four RCTs. Two studies reported time to complete healing (defined by complete corneal re-epithelialisation with clearance of infiltrate and hypopyon). Although this serves as a good outcome measure, it is important to bear in mind that some eyes with IK may develop delayed corneal epithelial wound healing or persistent epithelial defect after complete sterilisation of the ulcer in relation to neurotrophic keratopathy. Interestingly a transient increase in hypopyon may be observed within 24 hours after PACK-CXL,

likely attributed to the significant death of microorganisms and release of endotoxins, similar to a Jarisch-Herxheimer reaction (Said et al., 2014). In addition, potential confounding factors such as age and status of diabetes may affect the corneal wound healing time (Bikbova et al., 2018, Chen et al., 2009, Dua et al., 1994). Visual outcome was examined in three RCTs; however, this parameter is often not the best outcome measure for IK because it can be confounded by the location (e.g. whether the visual axis is affected) and the severity of the corneal ulcer, which may vary significantly between cases.

Other important issue related to the evaluation of the efficacy of PACK-CXL was that some studies included deep corneal ulcers/infiltrates which were outside the Dresden protocol therapeutic window of 400 μm of the cornea. Spoerl et al. (2007) reported that 94% of the UVA was absorbed in the anterior 400 μm of the cornea, therefore deeper ulcers are unlikely to benefit from adjuvant PACK-CXL. In addition, some studies included eyes with corneal thickness of <400 μm, which could potentially increase the risk of endothelial dysfunction following PACK-CXL (Chen et al., 2015). Anterior segment optical coherence tomography would better quantify the depth of ulcer and the corneal thickness than slit lamp examination or ultrasound pachymetry for eyes with IK as these two parameters can be highly variable. It is also noteworthy to mention that the 400 μm limit is based on CXL studies on healthy corneas but not on inflamed or infected corneas. It is likely that the transmission of UV light behaves differently in infected corneas (Abbouda et al., 2018) but further research studies are required to elucidate this aspect. Corneal densitometry using Pentacam Scheimpflug imaging system may also serve as a useful adjuvant tool for monitoring the corneal response to IK (Otri et al., 2012a). Many studies reported the application of fluorescein stain prior to PACK-CXL to measure the size of the epithelial defect. This has an important clinical implication because the presence of fluorescein could compete with riboflavin for energy at 365 nm and reduce the effectiveness of PACK-

CXL (Richoz et al., 2013). However, it is not always possible to ascertain the interval between the application of fluorescein drop and the initiation of PACK-CXL.

Based on the available evidence, three RCTs demonstrated that adjuvant PACK-CXL could expedite the resolution of IK in bacterial keratitis and potentially fungal keratitis, in terms of size of infiltrates (Bamdad et al., 2015, Kasetsuwan et al., 2016) and time taken to complete healing (Said et al., 2014), though recent studies did not demonstrate any additional benefit of PACK-CXL for fungal keratitis (Prajna et al., 2020, Ting et al., 2020d). There were insufficient data to perform any meaningful analysis for Acanthamoeba, viral and mixed IK. Several *in vitro* studies have shown that PACK-CXL did not confer any positive anti-amoebic effect on Acanthamoeba cyst or trophozoites (Atalay et al., 2018, del Buey et al., 2012). Interestingly, when riboflavin was substituted with rose bengal, PACK-CXL was shown to demonstrate effective anti-amoebic activity (Atalay et al., 2018). Based on histopathologic examination, Hager et al. (2016) similarly reported the persistence of Acanthamoeba cyst and trophozoites in the Acanthamoeba-infected corneal buttons after combined PACK-CXL and cryotherapy. PACK-CXL should not be employed to treat viral keratitis as UV radiation may exacerbate or activate herpes simplex infection (Price et al., 2012, Kymionis et al., 2007).

It is noteworthy to mention that while there was a statistically significant reduction of the healing time by 7 days after adjuvant PACK-CXL, the benefit of the procedure needs to be balanced by the availability of workforce, cost of the procedure, and healthcare resources for accommodating the surgical procedure, particularly when this procedure is usually performed as an emergency procedure. In light of the current evidence, it is recommended that adjuvant PACK-CXL is performed in bacterial and/or superficial fungal keratitis that are not refractory to medical treatment.

5.4.3. Quality of evidence and potential biases in the review

All four RCTs were associated with a high risk of performance bias (the participants and investigators were not masked from the procedure) and at least one or more high / unclear risk of bias in other domains. The overall quality of the evidence was judged to be very low (see **Table 5.2**). As such, further research is very likely to have an important impact on the confidence in the estimate of effect and is likely to change the estimate. The main reasons for downgrading the evidence included risk of bias in the included studies, inconsistency, imprecision and indirectness. Other important risk of bias included the lack of prospective registration of the clinical trial in a publicly accessible database (Bamdad et al., 2015, Said et al., 2014) and the presence of conflict of interest (Said et al., 2014). As fewer than 10 studies were eligible for inclusion, it was not possible to use a funnel plot to identify any publication bias.

In summary, adjuvant PACK-CXL may serve as a useful addition to the therapeutic armamentarium for IK in reducing the time to complete healing and size of infiltrate. There remains uncertainty regarding the effectiveness and safety of adjuvant PACK-CXL in the treatment of fungal keratitis and its use was cautioned in severe deep fungal cases. The use of PACK-CXL in Acanthamoeba keratitis remains elusive, with contradicting evidence from *in vitro* and clinical studies, whereas PACK-CXL is contraindicated in cases of viral keratitis. Further adequately powered, high-quality RCTs are required to provide a true evaluation of the effectiveness and safety of PACK-CXL. Standardisation of the reporting of outcome measures will enable better applicability of the evidence and allow easier comparison of the results across different studies related to PACK-CXL.

CHAPTER 6

Amniotic Membrane Transplantation for Infectious Keratitis: A Systematic Review and Meta-Analysis

6.1. Introduction

Infectious keratitis (IK) is the leading cause of corneal blindness worldwide (Ting et al., 2021j). Broad-spectrum topical antimicrobial therapy is currently the gold standard for managing IK in routine clinical practice, though eye-saving procedures such as corneal gluing, photoactivated chromophore-corneal collagen cross-linking (PACK-CXL), amniotic membrane transplant (AMT) and therapeutic keratoplasty may be required in recalcitrant cases (Dua et al., 2004, Tuli et al., 2007, Ting et al., 2019e, Hossain et al., 2018).

Liu et al. (2019) recently conducted a systematic review of 17 studies on the use of AMT for infective and non-infective corneal ulcers. Although the review provided a detailed analysis on the corneal healing rate and visual improvement rate associated with the use of AMT, it did not compare the effect of adjuvant AMT and SAT with SAT alone. Furthermore, the study did not include several important relevant studies, including three randomised controlled trials (RCTs) (Tabatabaei et al., 2017, Arya et al., 2008, Zeng et al., 2014) and one non-randomised controlled study (NRCS) (Kheirkhah et al., 2012), rendering the robustness of evidence uncertain. In light of these limitations, this systematic review and meta-analysis aimed to critically examine the effectiveness and safety of combined AMT and SAT versus SAT alone in treating patients with IK.

6.2. Methods

6.2.1. Protocol registration

The systematic review protocol was registered with PROSPERO (registration number: CRD42020175593) and The Joanna Briggs Institute of Evidence Synthesis (Ting et al., 2020b).

6.2.2. Data sources and search methods

Two authors (DSJT and CH) searched MEDLINE (January 1950 to November 2020), EMBASE (January 1980 to November 2020), Cochrane Central Register of Controlled Trials (CENTRAL), ISRCTN registry (www.isrctn.com/), US National Institutes of Health Ongoing Trials Register ClinicalTrials.gov (http://clinicaltrials.gov) and World Health Organisation (WHO) International Clinical Trials Registry Platform (ICTRP) (www.who.int/ictrp) for primary research related to AMT for IK. There was no date or language restriction in the search for trials. Electronic databases were first searched on 01 March 2020, followed by a final update on 01 November 2020. Key words used were “amnion”, “amniotic membrane”, “corneal ulcer”, “corneal infection”, “infectious keratitis”, and “microbial keratitis”. Search strategies for MEDLINE and EMBASE are provided in the **Appendix 3**.

6.2.3. Study selection

All clinical studies, encompassing RCTs, NRCSs and case series (n ≥ 5 eyes), related to AMT for IK were included as few RCTs were anticipated. The analysis was conducted at two levels; (1) a meta-analysis of all eligible RCTs and (2) a systematic review of all clinical studies, including NRCSs and case series. All types of IK, including bacterial, fungal, viral, parasitic or mixed infection, were included in this review. Studies that evaluated cases of non-infectious keratitis, other types of surgical

interventions, or AMT used for non-antimicrobial purpose were excluded from this study. Small case series (N < 5 eyes), reviews, published abstracts, laboratory and animal studies were also excluded. For the meta-analysis, the intervention group included cases of IK that were treated by AMT and SAT whereas the control group included cases of IK that were treated with SAT alone. There was no restriction applied to the published language, location or setting of the study, or patient demographic factors. This study conformed to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) guideline (Moher et al., 2009).

6.2.4. Data extraction

A web application designed for systematic reviews, Rayyan (Qatar) (Ouzzani et al., 2016), was used to help collate the potential studies and expedite the initial screening of abstracts and titles. The titles and abstracts obtained from the searches were independently screened by two authors (DSJT and CH) to include studies that fulfilled the eligibility criteria. Both authors then independently assessed the full-text version of all the selected articles and extracted data onto a standardised data collection form for qualitative review. The extracted data included the authors, year of publication, sample size, types of interventions, types of causative microorganisms, outcomes and complications. Discrepancies were resolved by group consensus and independent adjudication (HSD) if consensus could not be reached.

For the meta-analysis, the following information were extracted from the included RCTs and entered into RevMan (Review Manager 5.4) software (Review Manager (RevMan) Version 5.4.1, 2020):

(3) Study characteristics: Year of publication, country of study, prospective registration of clinical trials in a publicly accessible database, sample size, eligibility criteria, demographic factors, diagnostic criteria, method of

randomisation, method of masking, number of study arms, number of participants, types and techniques of AMT, types of comparators, source of funding, and any potential conflict of interest. Types of AMT were divided based on the preservation technique, which included fresh, cryopreserved, freeze-dried (or lyophilisation), air-dried and others. Surgical techniques were categorised based on the number of AMT (i.e. single vs. double vs. multiple layers) and the type of transplant (i.e. overlay/patch vs. inlay/graft vs. mixed overlay and inlay).

(4) Outcomes: Primary and secondary outcomes (including the time point at which the outcomes were assessed), intra- and post-operative complications, adverse events, need for secondary surgery, duration of follow-up, and rate of loss to follow-up.

6.2.5. Outcome measures

For the meta-analysis, the primary outcome measure was the time to complete corneal healing (defined as complete corneal re-epithelialisation and clearance of infiltrate and hypopyon; days) and the secondary outcome measures included the corrected-distance-visual-acuity (CDVA; logMAR) and uncorrected-distance-visual-acuity (UDVA; logMAR) at final follow-up (1-6 months), size of corneal ulcer (at 1-6 months), extent of corneal vascularisation, and risk of adverse events (defined as worsening IK and/or corneal melt/perforation requiring gluing or tectonic/therapeutic keratoplasty or evisceration) at final follow-up (1-6 months). These outcome measures were similarly used in the previous systematic review and meta-analysis in examining the effectiveness and safety of adjuvant PACK-CXL for IK (Ting et al., 2019e). Relevant data of all included studies were summarised and reported.

Continuous variables such as time to complete corneal healing, UDVA, CDVA, and size of corneal epithelial defect and infiltrate, were presented as mean with standard deviation (SD). In studies that reported median and interquartile range, the means and SDs were estimated using formulas reported by Wan et al. (2014) and the Cochrane Handbook estimator (Higgins and Green, 2011a). Dichotomous variable such as risk of adverse events was defined by the number of eyes with adverse events.

6.2.6. Assessment of risk of bias

Risk of bias was assessed by two authors (DSJT and CH) independently and any disagreement was adjudicated by HSD. Included RCTs were assessed for sources of systematic bias using the revised Cochrane risk of bias tool (RoB 2 tool) (Sterne et al., 2019). Both review authors were not masked to the authors of the studies during this assessment. A judgement of risk of bias as 'high risk', 'some concern', or 'low risk' was made for the following domains: (1) randomisation process; (2) deviations from intended interventions; (3) missing outcome data; (4) measurement of the outcome; and (5) selection of the reported result (Sterne et al., 2019). NRCSs were assessed for risk of bias using the ROBINS-I tool (Sterne et al., 2016b) against seven domains; the worst judgement in any of the domains was used as the overall risk of bias.

6.2.7. Measure of treatment effect

Dichotomous data were measured as risk ratios (RRs) with 95% confidence intervals (CI) and continuous data as mean differences (MDs) with 95% CI. The unit of analysis was the eye as the patient might have different causes of infection and treatment in each eye. There was no issue with the unit of analysis in the included RCTs. The review was conducted based on the available data from the trials. When data were unavailable but the level of missing data and reasons for missing data in each group

were similar, data were analysed even when intention-to-treat (ITT) analysis was not performed.

6.2.8. Assessment of heterogeneity

The heterogeneity of the RCTs and NRCSs was checked by careful review of the full-text, assessment of forest plots and examination of the I^2 value with its confidence interval. The overall characteristics of the studies, in particular the types of participants, causes of IK and types of interventions were examined to assess the extent to which the studies were similar enough to make pooling study results sensible. The results of forest plots were reviewed for consistency of the size and direction of effects. I^2 values greater than 50% were considered indicative of substantial heterogeneity and meta-analysis could not be conducted due to inconsistency of effect estimates (Higgins and Green, 2011a). Random-effects model was used for the meta-analysis as some degree of heterogeneity will always exist due to clinical and methodological differences of the studies. The Chi² p-value was also considered as this has a low power when the number of studies were few. A p-value of <0.1 was considered statistically significant (Higgins and Green, 2011a).

6.2.9. Data synthesis and analysis

A meta-analysis was undertaken when there were sufficient similarities in the reporting of outcome measures. A random-effects model in RevMan 5.4 was used in view of the expected heterogeneity across different studies. The Mantel-Haenszel method was employed for analysing the risk ratio (RR) of adverse events in view of the small expected number of events. If there was inconsistency between the results of individual studies such that a pooled result might not be a good summary of the individual trial results – for example, the effects were in different directions or I^2 >50% and P <0.1 – the data were not pooled but described in narrative format. Where there

was statistical heterogeneity, the data were pooled when all the effect estimates were in the same direction, such that a pooled estimate would seem to provide a good summary of the individual trial results. Sensitivity analysis was performed by assessing the impact of including studies at high risk of bias for an outcome in one or more key domains. This was conducted by omitting each study in turn to examine the influence of individual studies (with high risk of bias) on the overall pooled estimate. A summary of findings is presented below including the assessment of the quality of the evidence for outcomes using the GRADE approach with GRADE Pro/GDT software (Schunemann et al., 2013). All RCTs were started with a rating of 'high-quality' evidence and were downgraded by one level for serious concerns (or by two levels for very serious concerns) regarding the risk of bias, inconsistency, indirectness, imprecision and publication bias. The quality of evidence of studies was graded by two assessors (DSJT and CH) independently and any disagreement was adjudicated by HSD.

6.2.10. Subgroup analysis based on the type of organisms

In addition to the meta-analysis, a subgroup analysis of the effectiveness and adverse events based on different types of organisms was performed. In view of the anticipated low number of RCTs, both experimental and quasi-experimental study designs including RCTs and NRCSs were included in the subgroup analysis. Descriptive case series were also reviewed for rare or uncommon adverse events. Pooled estimates of the time to complete corneal healing and risk of adverse events across comparative studies, including RCTs and NRCSs, were calculated. Causes of IK were categorised as bacterial, fungal, viral, Acanthamoeba, or mixed infection.

6.3. Results

6.3.1. Literature search and study characteristics

The electronic searches last conducted on 01 November 2020 retrieved a total of 969 titles and abstracts (see **Figure 6.1** for the PRISMA flow chart). After removing 262 duplicates and including three additional records identified through other sources, the remaining 709 records were screened and 669 references that were not relevant to the scope of the review were excluded. A total of 35 full-text copies of papers were assessed for eligibility. After excluding seven ineligible articles, 28 studies were included in the systematic review. These included four RCTs (Arya et al., 2008, Li et al., 2014b, Tabatabaei et al., 2017, Zeng et al., 2014), three NRCSs (Kheirkhah et al., 2012, Li et al., 2014a, Naeem et al., 2014), and 21 case series (Altay et al., 2016, Berguiga et al., 2013, Bourcier et al., 2004, Chen et al., 2006, Chen et al., 2002, Eleiwa et al., 2020, Eraslan Yusufoglu et al., 2013, Fu et al., 2012, Gicquel et al., 2007, Hoffmann et al., 2013, Kim et al., 2001, Li et al., 2010, Mohan et al., 2014, Rao et al., 2012, Shi et al., 2007, Spelsberg and Reichelt, 2008, Wan and Huo, 2010, Wu et al., 2013b, Xie et al., 2014, Yildiz et al., 2008, Zhang et al., 2010), examining the efficacy and safety of AMT in 861 eyes with IK, of which 666 eyes received combined AMT with SAT and 195 eyes received SAT alone.

The causative microorganisms in the treatment group of AMT with SAT included 240 (36.0%) fungi, 199 (29.9%) bacteria, 152 (22.8%) herpes viruses, 9 (1.4%) Acanthamoeba, 18 (2.7%) mixed, and 48 (7.2%) unspecified IK (either bacteria, fungi or mixed infection). The main characteristics of all RCTs and NCRSs, including the authors' name, year of publication, number of treated eyes, types of causative microorganisms, severity of IK, types and techniques of AMT, main outcomes, and adverse events are summarised in **Table 6.1** and **Table 6.2**, respectively. Outcomes of RCTs are analysed and summarised under the meta-analysis section.

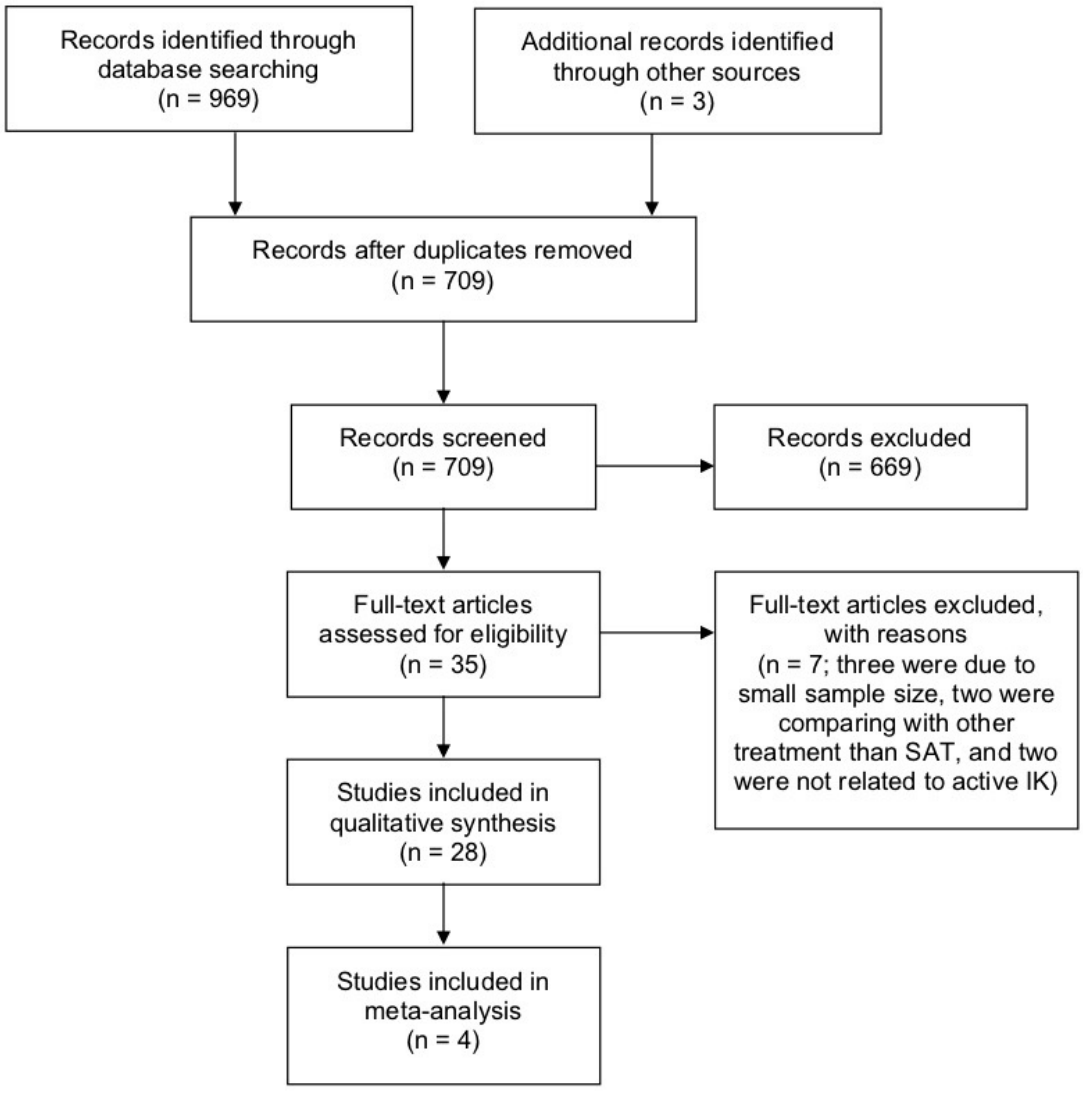


Figure 6.1. PRISMA flow diagram of the literature search for AMT for IK.

Table 6.1. Summary of RCTs on amniotic membrane transplant for infectious keratitis.

Table 6.1. Summary of all randomised control trials evaluating the effectiveness and safety of adjuvant amniotic membrane transplant (AMT) for infectious keratitis.

Authors	Year	Age, years	Male gender	Total eyes (AMT)	Total eyes (control)	Causative organisms (in AMT group)				
						B	F	A	V	M
Arya et al.	2008	Mean = 41.8 (AMT) vs. 50.8 (control)	33 (83%)	20	20	5	12	0	0	3
Li et al.	2014	Mean = 43.8 (AMT) vs. 47.0 (control)	36 (72%)	25	25	0	25	0	0	0
Tabatabaei et al.	2017	Mean = 48.3 (AMT) vs. 43.4 (control)	46 (46%)	49	50	49	0	0	0	0
Zeng et al.	2014	Mean = 57.1 (AMT) vs. 54.0 (control)	14 (70%)	10	10	0	10	0	0	0

Authors	Baseline vision (LogMAR)	Time from first presentation to AMT	AMT technique	Severity of ulcer[$]	Follow-up
Arya et al.	<20/400 = 80% (AMT) vs. 65% (control)	NR	Overlay (single layer)	2-5mm = 60% (AMT) vs. 45% (control)	3 months
Li et al.	<20/200 = 44% (AMT) vs. 48% (control)	2 days	Overlay (single or double layer)	NS	1 month
Tabatabaei et al.	Mean = 1.7 (AMT) vs. 1.8 (control)	2-5 days	Inlay (double layer)	Mean = 26mm^2 (AMT) vs. 27mm^2 (control)	6 months
Zeng et al.	Median = 1.0 (AMT) vs. 1.0 (control)	NR	Overlay (single layer)	Mean = 7mm (AMT) vs. 7mm (control)	1 month

B = Bacteria; F = Fungi; A = Acanthamoeba; V = Viruses; M = Mixed infection; NR = Not reported

[$]Severity of the corneal ulcer is presented either in maximum linear diameter (mm) or in area (mm^2).

Table 6.2. Summary of non-RCT studies on amniotic membrane transplant for infectious keratitis.

Table 6.2. Summary of all clinical studies (excluding RCTs) evaluating the use of amniotic membrane transplant (AMT) for infectious keratitis.

Authors	Year	Study design	Total eyes (AMT)	Total eyes (control)	Causative organisms (in AMT group)					Severity of ulcer[$]
					BK	FK	VK	AK	MK	NSK	
Kheirkhah et al.	2012	NRCS	14	11	14						Mean = 32% (AMT) vs. 33% (control)
Li et al.	2014	NRCS	53	45		53					Not reported
Naeem et al.	2004	NRCS	34	34						34	>3mm
Altay et al.	2016	Case series	84	-	42		42				3mm
Berguiga et al.	2013	Case series	5	-	1		4				Central, deep ulcer / perforation (<2mm)
Bourcier et al.	2004	Case series	6	-				6			Stromal lesions
Chen et al.	2006	Case series	23	-		12			11		2-13mm; >50% depth to perforation
Chen et al.	2002	Case series	6	-	6						Mean = 29mm^2, depth of 25-33% (50%), descemetocele (50%)
Eleiwa et al.	2020	Case series	5	-		5					Perforated corneal ulcer (3-5mm)
Eraslan Yusufoglu et al.	2013	Case series	46	-	21		25				Paracentral and central ulcer; depth of >50% in viral (56%) and bacterial (33%) cases
Fu et al.	2012	Case series	35	-		35					Median = 3-5mm
Gicquel et al.	2007	Case series	12	-	12						Mean = 5mm
Hoffmann et al.	2013	Case series	12	-	3		5		4		4-20mm^2, depth of 10-90%
Kim et al.	2001	Case series	21	-	9	2	7	3			Not reported
Li et al.	2010	Case series	18	-			18				<5mm (67%), >5mm (33%), perforation (6%)
Mohan et al.	2014	Case series	28	-	28						Not reported
Rao et al.	2012	Case series	21	-		21					Not reported
Shi et al.	2007	Case series	15	-			15				20-45mm^2, depth of 20-33%
Spelsberg et al.	2008	Case series	12	-			12				-
Wan & Huo	2010	Case series	35	-	9	20	6				Not reported
Wu et al.	2013	Case series	18	-			18				1-7mm
Xie et al.	2014	Case series	19	-		19					3-6mm
Yildiz et al.	2008	Case series	14	-						14	Not reported
Zhang et al.	2010	Case series	26	-		26					Median = 3-6mm; 5 perforation

Table 6.2 (Continue). Summary of all clinical studies (excluding RCTs) evaluating the use of amniotic membrane transplant (AMT) for infectious keratitis.

Authors	Preop vision*	Time to AMT	AMT technique**	Combined with SAT	Outcomes (i.e. healing rate and time)	Adverse event***	Postop vision*	Follow-up
Kheirkhah et al.	2.0 (AMT) vs. 2.0 (control)	2-3 days	Overlay (Single)	Y	Complete healing = 100%, Mean healing time = 13 days (AMT) vs. 16 (control)	None	Mean = 0.5 (AMT) vs. 0.7 (control)	11 months
Li et al.	0.6	Not reported	Inlay	Y	Mean healing time = 23 days (AMT) and 35 days (control)	Not reported	Mean = 0.3 (AMT) vs. 0.4 (control)	2 months
Naeem et al.[35]	Not reported	Not reported	Not reported	Y	Complete healing = 96% (AMT) vs. 30 (87%) (control)	Not reported	Not reported	2 months
Altay et al.[36]	Majority ≤1.0	2-5 days	Overlay (Single or double)	Y	Complete healing = 100% (BK) and 95% (VK), Mean healing time = 19 days	5% (VK)	Improved in vision = >50% (BK); 2% (VK)	15 months
Berguiga et al.[37]	Mean = 1.0	7 days	Mixed (Multiple)	Y	Complete healing = 100% (BK) and 75% (VK)	25% (VK)	Improved in 40% cases	15 months
Bourcier et al.[38]	All ≤CF	Not reported	Inlay or overlay (Single – multiple)	Y	Complete healing = 67%	None	Improved in 50%	14 months
Chen et al.[39]	CF – LP	Not reported	Inlay (Single or double)	Y	Complete healing = 87%, Mean healing time = 16 days	13%	6/6 - LP	21 months
Chen et al.[40]	HM – LP	21 days	Inlay (Single)	Y	Complete healing = 83%, Mean healing time = 9 days	17%	Improved in 83%	13 months
Eleiwa et al.[41]	Median = CF	12 days	Inlay (Double)	Y	Complete healing = 100%, Mean healing time = 26 days	None	Median = 0.3	14 months
Eraslan Yusufoglu et al.[42]	Median = ≤1.0	Not clear	Overlay (Double)	Y	Complete healing = 100% (BK), 88% (VK), Mean healing time = 23 days	12% (VK)	Improved in 57% (BK) and 8% (VK)	12 months
Fu et al.[43]	Median = <1.0	Not reported	Overlay (Double)	Y	Complete healing = 94%, Mean healing time = 14 days	3%	Improved in 94%	12 months
Gicquel et al.[44]	Median = ≤1.0	2 days	Overlay or mixed (Single – multiple)	Y	Complete healing = 100%, Mean healing time = 26 days	None	Median = 0.3	8 months
Hoffmann et al.[45]	Median = 6/6000	Not reported	Mixed (Multiple)	Y	Complete healing = 83%, Mean healing time = 24 days	17%	Median = 1.0	22 months

Kim et al.[46]	0.3 – LP	Not reported	Inlay or overlay (Single – multiple)	Y	Complete healing = 100%	None	Improved in 76%	18 months
Li et al.[47]	~0.8	~1 week	Overlay (Double)	Y	Complete healing = 100%	None	Improved in 78%	3-18 months
Mohan et al.[48]	<1.0	4 weeks	Mixed (Multiple)	Y	Complete healing = 75%	25%	Improved in 7%	6 months
Rao et al.[49]	Not mentioned	~2 weeks	Mixed (Multilayer)	Y	Complete healing = 76%	10%	Not reported	3 months
Shi et al.[50]	Median = 1.3	2 weeks	Overlay (Multiple)	Y	Complete healing = 100%, Mean healing time = 15 days	None	Median = 0.3 Improved in 93%	9 months
Spelsberg et al.[51]	Median = <1.0	Not reported	Inlay (Single)	Y	Complete healing = 75%, Mean healing time = 25 days	25%	Improved in 8%	7 months
Wan & Huo[52]	Not reported	Not reported	Mixed (Multiple)	Y	Complete healing = 94%	6%	>1.0 = 83%	1-2 months
Wu et al.[53]	>1.0 = 7 (39%)	1-3 days	Mixed (Multiple)	Y	Complete healing = 100%, Mean healing time = 17 days	None	>1.0 = 72%	4 months
Xie et al.[54]	Mean = 1.3	1 week	Mixed (Double)	Y	Complete healing = 42%, Mean healing time = 36 days	Not reported	Mean = 0.9	3 months
Yildiz et al.[55]	Not reported	Not reported	Overlay (Single)	Y	Complete healing = 100%	Not reported	Not reported	22 months
Zhang et al.[56]	Not reported	Not reported	Mixed (Double)	Y	Complete healing = 81%	8%	Improved in 80%	3-36 months

BK = Bacterial keratitis; FK = Fungal keratitis; VK = Viral keratitis; AK = Acanthamoeba keratitis; MK = Mixed keratitis; NSK = Non-specified keratitis; SAT = Standard antimicrobial treatment; NRCS = Non-randomized controlled studies; CF = Counting fingers; HM = Hand movement; PL = Perception of light

$Severity of the corneal ulcer is presented either in maximum linear diameter (mm), in area (mm^2) or in percentage of total cornea (%).

*Vision is presented in either Snellen vision or logMAR vision.

**AMT technique is categorized by the type of grafting (inlay as graft vs. overlay as patch vs. mixed inlay / overlay) and number of layers (single layer vs. double layer vs. multiple layers)

***Adverse event was defined as uncontrolled / worsening infectious keratitis requiring tectonic keratoplasty or evisceration.

6.3.2. Meta-analysis of eligible RCTs

6.3.2.1. Overall description

Four eligible RCTs were included in the meta-analysis, which included a total of 209 eyes that compared the effectiveness of SAT and adjuvant AMT with SAT alone (Arya et al., 2008, Li et al., 2014b, Tabatabaei et al., 2017, Zeng et al., 2014). These consisted of four single-centre RCTs conducted separately in China (n=2) (Li et al., 2014b, Zeng et al., 2014), India (n=1) (Arya et al., 2008), and Iran (n=1) (Tabatabaei et al., 2017). Two additional RCTs were also identified but were excluded from the meta-analysis as one RCT compared AMT with conjunctival flap (Abdulhalim et al., 2015) and another RCT compared AMT and argon laser treatment with AMT for treating IK (Khater, 2017). There was no ongoing trial identified from the clinical trial registry databases. The RCTs included participants with an average age between 45.5 and 55.6 years (ranged, 15-98 years) with a slight (61.7%) male preponderance. None of the RCTs were prospectively registered with any clinical trial database.

6.3.2.2. Types of microbes and severity of IK

The four RCTs (n=209 eyes) included a total of 108 (51.7%) eyes with bacterial keratitis, 95 (45.5%) eyes with fungal keratitis and 6 (2.9%) eyes with mixed bacterial / fungal keratitis. No herpetic or Acanthamoeba keratitis was included in any of the RCTs. Li et al. (2014b) and Zeng et al. (2014) included only fungal keratitis and Tabatabaei et al. (2017) included only bacterial keratitis whereas Arya et al. (2008) included a mixed cohort, which included bacterial, fungal or mixed bacterial / fungal keratitis. The proportion of the types of microorganisms was similar between the treatment and control groups among all RCTs.

The baseline reporting and extent of the severity of IK were heterogeneous among the four RCTs. Two studies reported the baseline ulcer diameter or area in continuous

values (Tabatabaei et al., 2017, Zeng et al., 2014), one study reported the baseline severity in categorical values (i.e. 1-2 mm, 2-5 mm, or >5 mm in diameter) (Arya et al., 2008), and one study did not report the baseline severity of IK (Li et al., 2014b). Tabatabaei et al. (2017) and Zeng et al. (2014) included IK cases with a mean ulcer diameter of 5.2 mm and 6.7 mm, respectively (i.e. severe IK based on previous studies) (Chidambaram et al., 2016, Stapleton et al., 2012). Similarly, Arya et al. (2008) included mainly moderate-to-severe IK, with 47.5% of moderate ulcer (2-5 mm in diameter) and 42.5% of severe ulcer (> 5mm in diameter). None of the RCTs included eyes presented with threatened or actual corneal perforation.

6.3.2.3. Surgical technique and timing of AMT

In terms of the preservation technique, two RCTs utilised cryopreserved AMs (Arya et al., 2008, Zeng et al., 2014), one RCT utilised freeze-dried AMs (Li et al., 2014b), and one did not report the type of AMs used. Three RCTs (Arya et al., 2008, Li et al., 2014b, Zeng et al., 2014) employed AMT as an overlay patch (single- or double-layer) whereas one RCT (Tabatabaei et al., 2017) employed AMT as an inlay graft (double-layer). All AMTs were performed with the epithelium / basement membrane side up. The time interval between the initial presentation of IK and AMT was 3 days in one study,(Li et al., 2014b) 2-5 days in one study (Tabatabaei et al., 2017), and unspecified in two studies (Arya et al., 2008, Zeng et al., 2014).

6.3.2.4. Risk of bias

The risk of bias of the RCTs, based on the five abovementioned domains, is summarised in **Figure 6.2**. Only one RCT (Tabatabaei et al., 2017) clearly reported the randomisation process, with details on randomisation and allocation concealment. All RCTs had complete or nearly complete (>99%) follow-up data. There were some concerns about the measurement of the outcome as it was not possible to mask the

outcome assessor (i.e. AMT would be visible on postoperative clinical examination, hence revealing the intervention arm). In addition, there were some concerns about selective reporting of outcomes across all RCTs as none of them were prospectively registered with any publicly accessible clinical trial database; therefore, the risk of bias could not be confidently evaluated. No relevant conflict of interest was declared in any of the RCTs.

The risk of bias of NRCS was assessed using the ROBIN-I tool. Two NRCS (Kheirkhah et al., 2012, Naeem et al., 2014) were judged to be at serious risk of bias due to bias in selection of participants and measurement of outcomes. One NRCS (Li et al., 2014a) was judged to be at critical risk of bias due to baseline confounding, bias in selection of participants and measurement of outcomes.

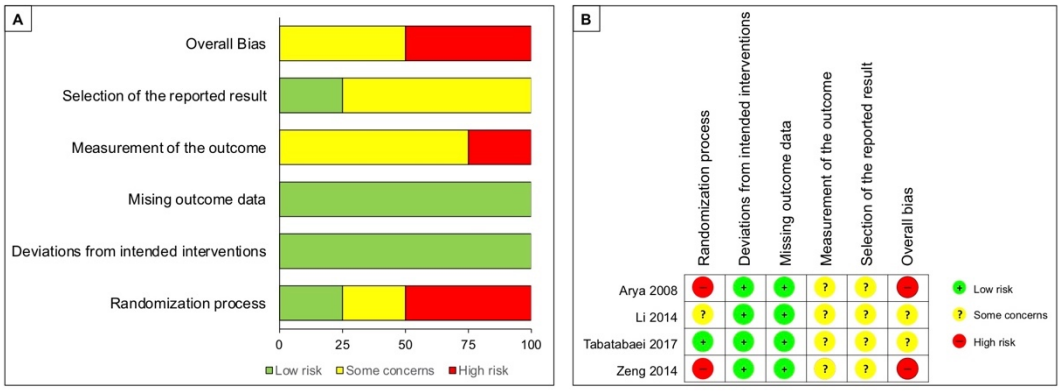


Figure 6.2. Risk of bias assessment of all RCTs on AMT for IK.
Risk of bias assessment was performed based on revised risk of bias tool (RoB 2). **(A)** A summary of review authors' judgements about each risk of bias item presented as percentages across all included RCTs. **(B)** Review authors' judgements about each risk of bias item presented individually for all included RCTs.

6.3.2.5. Effects of intervention

The effects of interventions were categorised into: (A) time to complete corneal healing (defined by complete corneal re-epithelialisation and resolution of infection); (B) UDVA and CDVA (in logMAR) at 1-6 months follow-up; (C) size of ulcer or infiltrate; (D) extent of corneal neovascularisation at 1-6 months; and (E) adverse events defined by worsening IK, endophthalmitis or corneal perforation requiring corneal gluing, tectonic keratoplasty or evisceration during the follow-up period. The GRADE summary of findings for each treatment outcome is summarised in **Table 6.3**.

6.3.2.5.1. Time to complete corneal healing

Three RCTs (n=169 eyes) reported the primary outcome measure, which was the time to complete corneal healing (Li et al., 2014b, Tabatabaei et al., 2017, Zeng et al., 2014). There is very low-quality evidence that adjuvant AMT expedited the time to complete corneal healing compared to SAT alone [mean difference (MD) -4.08 days; 95% CI -6.27 to -1.88; I^2=18%; p<0.001; **Figure 6.3A**]. The quality of evidence was downgraded due to the high risk of bias and imprecision. The randomisation process was not clear in one RCT (Zeng et al., 2014) and allocation concealment were not performed in two RCTs (Li et al., 2014b, Zeng et al., 2014). Furthermore, there was lack of blinding of the participants and the assessors across all three RCTs (Li et al., 2014b, Tabatabaei et al., 2017, Zeng et al., 2014). In addition, the total number of participants/eyes pooled in the meta-analysis was less than the number generated by a conventional sample size calculation.

6.3.2.5.2. Uncorrected-distance-visual-acuity (UDVA)

Two RCTs (n=119 eyes) reported the UDVA at 1 month follow-up (Tabatabaei et al., 2017, Zeng et al., 2014). There is very low-quality evidence that adjuvant AMT resulted in better UDVA compared to SAT alone (MD -0.26 logMAR; -0.50 to -0.02;

I^2=0; p=0.04; **Figure 6.3B**). The quality of evidence was downgraded due to high risk of bias and imprecision. The randomisation process and allocation concealment were not clear in one RCT. There was lack of blinding of the participants and assessors and the sample size was limited in both RCTs. Only one RCT (n=99 eyes) reported the UDVA at 6 months follow-up, where the adjuvant AMT group achieved a better UDVA than the SAT alone group (1.34 ± 0.69 vs. 1.69 ± 0.54; p<0.001) (Tabatabaei et al., 2017).

6.3.2.5.3. Corrected-distance-visual-acuity (CDVA)

Only one RCT (n=99 eyes) reported the CDVA at 1-6 months follow-up, therefore meta-analysis was not possible (Tabatabaei et al., 2017). There is low-quality evidence that adjuvant AMT resulted in better CDVA (in logMAR) at 1 month (1.44 ± 0.75 vs. 1.74 ± 0.61; p<0.001), 3 months (1.23 ± 0.72 vs. 1.65 ± 0.56; p=0.007), and 6 months follow-up (1.13 ± 0.68 vs. 1.55 ± 0.59; p<0.001). This RCT evaluated only bacterial keratitis, but not other types of microorganism. The quality of evidence was downgraded due to risk of bias (lack of blinding) and imprecision (limited sample size).

6.3.2.5.4. Size of ulcer or infiltrate

There was no RCT that reported the size of ulcer or infiltrate. However, one RCT (n=99 eyes) reported the area of corneal scarring (in mm^2) at 6 months follow-up, which was found to better in the adjuvant AMT group than the SAT alone group (17.6 ± 5.7 vs. 22.8 ± 6.1; p<0.001) (Tabatabaei et al., 2017). The quality of evidence was downgraded due to some risk of bias (lack of blinding) and imprecision (limited sample size).

6.3.2.5.5. Corneal vascularisation

Only one RCT (n=99 eyes) reported the extent of corneal vascularisation, measured as % of the total area of corneal surface, at 1-6 months follow-up (Tabatabaei et al., 2017). There is low-quality evidence that adjuvant AMT resulted in less corneal vascularisation (%) at 1 month (5.0 ± 4.0 vs. 7.0 ± 5.0; p<0.001) and 6 months follow-up (2.0 ± 3.0 vs. 7.0 ± 6.0; p<0.001). The quality of evidence was downgraded due to some risk of bias (lack of blinding) and imprecision (limited sample size).

6.3.2.5.6. Adverse events

All four RCTs (n=209 eyes) reported the occurrence / risk of adverse events, defined as worsening of IK or perforation requiring corneal gluing or tectonic keratoplasty (Arya et al., 2008, Li et al., 2014b, Tabatabaei et al., 2017, Zeng et al., 2014). There is very low-quality evidence that there was no difference in the risk of adverse events between adjuvant AMT and SAT alone groups at 1-6 months follow-up (RR 0.80; 95% CI 0.46 to 1.38; I^2=0; p=0.42; **Figure 6.3C**). The risk of adverse events was noted to be considerably variable across four RCTs, with a risk ranging between 0.0-26.5% in the AMT group and 10.0-32.0% in the SAT group. Such heterogeneity was likely related to the variations in the patient cohort, types and severity of IK, and the standard treatment regimen employed among different studies. The quality of evidence was downgraded due to high risk of bias and imprecision. The follow-up duration was variable across studies, ranging from 1 month to 6 months. One RCT (n=50 eyes with fungal keratitis) reported the risk of secondary glaucoma, which was 4% (n=1 eye) in the AMT group and 36% (n=9 eyes) in the SAT group (p=0.011) (Li et al., 2014a). According to the CONSORT reporting of harms guidance, all four RCTs were judged to be of inadequate quality as the severity of adverse events and clinical sequalae were not clearly described (Arya et al., 2008, Li et al., 2014b, Tabatabaei et al., 2017, Zeng et al., 2014).

Table 6.3. GRADE summary of findings for adjuvant amniotic membrane transplant for infectious keratitis.

Patient or population: Infectious keratitis
Intervention: Adjuvant amniotic membrane transplant (AMT)
Comparison: Standard antimicrobial treatment (SAT)

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	№ of eyes (studies)	Certainty of the evidence (GRADE)
	Risk with SAT	Risk with AMT			
Time to complete healing (days)	10.2-30.7 days	MD 4.08 days shorter (6.27 shorter to 1.88 shorter)	-	169 (3 RCTs)	⊕◯◯◯ VERY LOW [a,b,c]
UDVA (logMAR) at 1 months	1.27-1.88 logMAR	MD 0.26 logMAR better (0.50 better to 0.02 better)	-	119 (2 RCTs)	⊕◯◯◯ VERY LOW [a,b,c]
CDVA (logMAR) at 6 months	1.55 logMAR	MD 0.43 logMAR better (0.68 better to 0.17 better)	-	99 (1 RCT)	⊕⊕◯◯ LOW [b,c]
Size of corneal scar (mm^2) at 6 months	22.8 mm^2	MD 5.17 mm^2 smaller (7.53 smaller to 2.8 smaller)	-	99 (1 RCT)	⊕⊕◯◯ LOW [b,c]
Corneal vascularization (%) at 6 months	7%	MD 4% smaller (6 smaller to 3 smaller)	-	99 (1 RCT)	⊕⊕◯◯ LOW [b,c]
Adverse events at 1-6 months	23 per 100	18 per 100 (11 to 32)	RR 0.80 (0.46 to 1.38)	209 (4 RCTs)	⊕◯◯◯ VERY LOW [a,b,c,d,e]

*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

CI: Confidence interval; MD: Mean difference; RR: Risk ratio; UDVA: Uncorrected-distance-visual-acuity; CDVA: Corrected-distance-visual-acuity;

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Explanations:
a. High risk of bias due to lack of randomization and allocation concealment in ≥ 50% of the included studies.
b. Potential risk of bias due to the lack of blinding in participants and assessors.
c. The total number of participants is less than the number generated by a conventional sample size calculation.
d. There are few events and the confidence interval includes appreciable benefit and harm.
e. Differences in final follow-up duration.

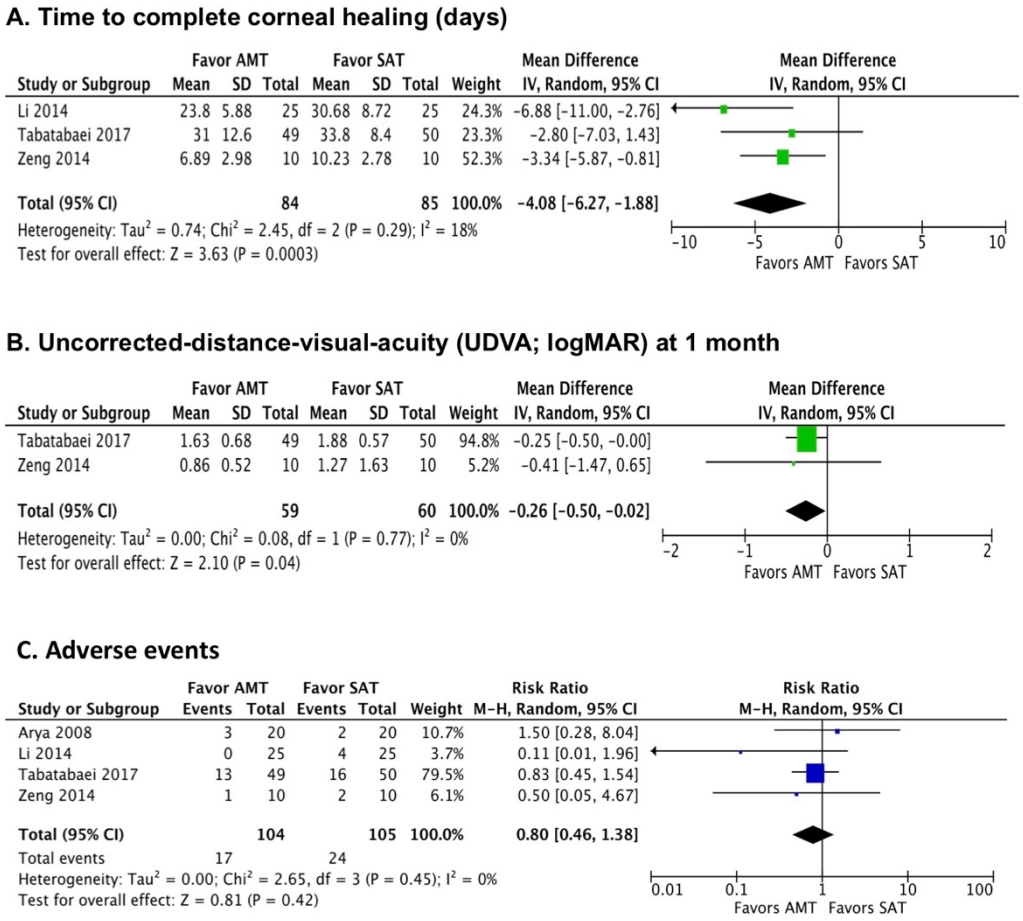


Figure 6.3. Forest plots on various outcomes of AMT for IK.

Summary of the meta-analysis (forest plot) comparing the efficacy between adjuvant amniotic membrane transplant (AMT) plus standard antimicrobial treatment (SAT) and SAT alone in eligible randomized controlled trials, in terms of: **(A)** time to complete corneal healing; **(B)** uncorrected-distance-visual-acuity; and **(C)** risk of adverse events.

6.3.3. Subgroup analysis based on the type of IK

There were insufficient RCTs to perform subgroup analysis between bacterial and fungal keratitis outcomes. In view of the limitation, pooled estimates on time to complete corneal healing and adverse events were assessed based on RCTs and NCRSs. Pooled estimates of the time to complete corneal healing showed that adjuvant AMT expedited the time to complete corneal healing in bacterial keratitis (MD -2.42 days; 95% CI -4.53 to -0.32; p=0.02) and fungal keratitis (MD -6.90 days; 95% CI -11.58 to -2.21; p=0.004; **Table 6.4**). In terms of the risk of adverse events,

the pooled estimates demonstrated that adjuvant AMT did not significantly influence the risk of adverse events in bacterial keratitis (RR 0.83; 95% CI 0.45-1.54), fungal keratitis (RR 0.28; 0.05-1.65), and mixed bacterial / fungal keratitis cohorts (RR 1.50; 0.28-8.04; **Table 6.4**).

Subgroup analysis of the outcomes based on large non-comparative case series (n≥5 eyes) is summarised in **Table 6.5**. Studies that reported the pooled results with no distinction made to the underlying microorganisms were excluded from the subgroup analysis. Based on these case series, complete corneal healing rate was 92.6% (113/122) in bacterial keratitis, 80.0% (96/120) in fungal keratitis, 93.8% (137/146) in herpetic keratitis, in 77.8% (7/9) Acanthamoeba keratitis, 81.8% (9/11) in mixed bacterial and fungal infection, and 100% (4/4) in mixed bacterial and herpetic infection.

Table 6.4. Pooled estimates of the time to complete corneal healing and risk of adverse events after adjuvant AMT for infectious keratitis.

IK Cohort	No. of studies	Types of studies	No. of eyes	Pooled estimates (95% CI)
Time to complete corneal healing (estimates presented in mean difference, MD)				
Bacterial	2	1 RCT 1 NRCS	124	-2.42 (-4.53 to -0.32)
Fungal	3	2 RCTs 1 NRCS	168	-6.90 (-11.58 to -2.21)
Risk of adverse events (estimates presented in risk ratio, RR)				
Bacterial	2	1 RCT 1 NRCS	124	0.83 (0.45 – 1.54)
Fungal	2	2 RCTs	70	0.28 (0.05 – 1.65)
Mixed bacteria and fungi	2	1 RCT 1 NRCS	108	1.50 (0.28 – 8.04)

IK = Infectious keratitis; CI = Confidence interval.

A negative MD value indicates that adjuvant AMT group has a shorter time to complete corneal healing compared to SAT alone group.

Table 6.5. Summary of the healing rate and treatment failure of AMT for infectious keratitis.

Authors	Year	Numbers	Complete healing	Healing time (days)	Adverse events
Bacterial keratitis					
Altay et al.	2016	42	42 (100%)	19	0 (0%)
Berguiga et al.	2013	1	1 (100%)	-	0 (0%)
Chen et al.	2002	6	5 (83%)	9	1 (17%)
Eraslan et al.	2013	21	21 (100%)	22	0 (0%)
Gicquel et al.	2007	12	12 (100%)	26	0 (0%)
Hoffmann et al.	2013	3	2 (67%)	31	1 (33%)
Kim et al.	2001	9	9 (100%)	-	0 (0%)
Mohan et al.	2014	28	21 (75%)	-	7 (25%)
Fungal keratitis					
Chen et al.	2006	12	11 (92%)	4 – 26	1 (8%)
Eleiwa et al.	2020	5	5 (100%)	26	0 (0%)
Fu et al.	2012	35	33 (92%)	14	1 (3%)
Kim et al.	2001	2	2 (100%)	-	0 (0%)
Rao et al.	2012	21	16 (76%)	-	2 (10%)
Xie et al.	2014	19	8 (42%)	36	Not reported
Zhang et al.	2010	26	21 (81%)	-	2 (8%)
Herpetic keratitis					
Altay et al.	2016	42	40 (95%)	19	2 (5%)
Berguiga et al.	2013	4	3 (75%)	-	1 (25%)
Eraslan et al.	2013	25	22 (88%)	22	3 (12%)
Hoffmann et al.	2013	5	5 (100%)	21	0 (0%)
Kim et al.	2001	7	7 (100%)	-	0 (0%)
Li et al.	2010	18	18 (100%)	-	0 (0%)
Shi et al.	2007	15	15 (100%)	15	0 (0%)
Spelsberg et al.	2008	12	9 (75%)	25	3 (25%)
Wu et al.	2013	18	18 (100%)	17	0 (0%)
Acanthamoeba keratitis					
Bourcier et al.	2004	6	4 (67%)	-	0 (0%)
Kim et al.	2001	3	3 (100%)	-	0 (0%)
Mixed bacterial and fungal keratitis					
Chen et al.	2006	11	9 (82%)	7 – 23	2 (18%)
Mixed bacterial and herpetic keratitis					
Hoffmann et al.	2013	4	4 (100%)	22	0 (0%)

6.4. Discussion

This study represents the most up-to-date systematic review and meta-analysis examining the effectiveness and safety of adjuvant AMT for treating IK. The role of AMT in ocular diseases was first documented in 1940 (De Rotth, 1940). However, it was not until the early 1990s that Batlle and Perdomo had reinvigorated the interest of employing AMT for various ocular surface diseases, for which they noted that the process of corneal re-epithelialisation could be achieved within 72 hours of surgery (Dua et al., 2004). Subsequently, Kim and Tseng (Kim and Tseng, 1995) popularised the use of AMT for ocular surface reconstruction using glycerin-preserved AM. Although AMT has since been performed to treat a wide array of ocular surface diseases, high-quality evidence of using AMT in treating active IK remains limited. So far, there has only been one systematic review that had partially examined the benefit of AMT in IK (Liu et al., 2019). The systematic review included 17 studies (n=390 eyes) and examined the healing rate and visual improvement rate after AMT in either infectious or non-infectious corneal ulcers. While the review showed that AMT was effective in treating IK, it did not answer a very important clinical question, which is whether adjuvant AMT provides any additional benefit or risk during the management of IK, in addition to standard antimicrobial treatment. In addition, the review did not capture 3 RCTs that were identified in this systematic review, rendering the evidence of their findings uncertain. Furthermore, the effect of AMT has not been examined in the context of the types of organisms.

6.4.1. Summary of main findings

In this systematic review, a total of 28 studies (n=861 eyes) were included, encompassing four RCTs with 209 eyes and 24 non-RCTs with 657 eyes. Among the RCTs, the majority of the included eyes were either affected by bacterial keratitis or fungal keratitis, with only 6 eyes being affected by mixed bacterial and fungal keratitis.

None of the RCTs examined the effect of adjuvant AMT in herpetic or Acanthamoeba keratitis. All the RCTs employed either single- or double-layer AMT for treating the IK. In addition, the AMT was performed during the acute and active stage of IK [with two studies (Arya et al., 2008, Zeng et al., 2014) providing specific timing of the AMT (i.e. 2-5 days from the initial presentation). In addition, all cases were treated empirically with broad-spectrum topical antimicrobial treatment initially as per the local guideline. Based on the meta-analysis of three RCTs, early adjuvant AMT was shown to significantly shorten the time to complete corneal healing by approximately 4 days when compared to SAT alone. In addition, it was shown that IK patients treated with adjuvant AMT achieved 0.26 logMAR (equivalent to 2-3 Snellen lines) better UDVA at 1 month follow-up compared to SAT alone. No difference was noted in terms of the risk of adverse events. Furthermore, other beneficial effects such as better CDVA and less corneal vascularisation were shown in the adjuvant AMT group.

The observed beneficial effects in the adjuvant AMT group are likely attributed to the dual antimicrobial and anti-inflammatory properties of AMT (Jirsova and Jones, 2017, Dua et al., 2004). The plausible mechanisms of antimicrobial effect of AMT in IK are at least twofold. First, the antimicrobial activity is directly linked to the presence of various antimicrobial components, including lysozyme, transferrin, and immunoglobulin, in the amniotic fluid (Galask and Snyder, 1970, Dua et al., 2004). Second, studies have shown that AM could serve as an effective antibiotic reservoir when used in combination with antibiotics and provide sustained drug delivery (Yelchuri et al., 2017). Furthermore, AM has been shown to exhibit anti-inflammatory function via regulation of T-cell function and secretion of anti-inflammatory antagonists, including IL-1ra, sTNF, and VEGF-R (Grzetic-Lenac et al., 2011) (Laranjeira et al., 2018). This anti-inflammatory property will have a long-term beneficial effect on the cornea, hence vision, as persistent corneal vascularisation could negatively affect the vision, either directly via encroachment on the visual axis

or indirectly via lipid keratopathy where exudation of lipid and inflammatory cells extends to the visual axis (Nicholas and Mysore, 2020). Moreover, it increases the risk of graft rejection should corneal transplantation need to be carried out to restore the optical clarity of the cornea (Faraj et al., 2014, Nicholas and Mysore, 2020).

Although the benefit of AMT in herpetic keratitis has not been ascertained in RCTs, this systematic review (based on large case series) showed that it enabled a high rate (94%) of complete corneal healing. Herpetic keratitis, particularly the necrotising form, is a potentially sight-threatening condition that is difficult to treat clinically (Sibley and Larkin, 2020). It is also notoriously known to be associated with neurotrophic keratopathy, which results in delay in corneal healing (Sibley and Larkin, 2020, Ting et al., 2019d). Therefore, AMT serves as a useful adjunct treatment in this clinical circumstance in view of its dual anti-viral and wound healing properties (Lee and Tseng, 1997, Mamede et al., 2012). On the other hand, only two case series have reported the use of AMT in Acanthamoeba keratitis, albeit good effect was observed. The broad-spectrum antimicrobial activity of AMT (observed in this systematic review) against a wide range of microorganisms is particularly beneficial in the management of IK as polymicrobial keratitis is a relatively common entity, which often poses significant diagnostic and therapeutic challenges (Khoo et al., 2020, Ting et al., 2019c, Ting et al., 2021h). Based on large case series, adjuvant AMT has been shown in this review to be an effective treatment for mixed bacteria/fungal keratitis (82% complete healing; n=9/11) and mixed bacterial/herpetic keratitis (100% complete healing; n=4/4).

A number of studies have also demonstrated that AMT, when employed as multi-layer, could effectively treat IK with threatened or actual corneal perforation (up to 5mm) (Chen et al., 2006, Eleiwa et al., 2020, Li et al., 2010, Zhang et al., 2010). Studies have demonstrated that multi-layer AMs can promote re-epithelialisation of

the cornea and integrate with the corneal stroma, with progressive repopulation of AM by cornea stroma-derived cells, ultimately resulting in corneal healing and thickening (Nubile et al., 2011). This approach helps obviate the need for an emergency therapeutic keratoplasty, which is known to be associated with high risk of graft failure (in the setting of active inflammation) and recurrence of infection (Ang et al., 2012, Moon et al., 2020). It would also help reduce the burden on the availability of donor corneas, which have been significantly affected by the recent COVID-19 pandemic (Thuret et al., 2020).

6.4.2. Overall completeness and applicability of evidence

This study represents the most up-to-date systematic review and meta-analysis specifically examined the effectiveness and safety of adjuvant AMT in managing all types of IK (Ting et al., 2021g). The four RCTs included primarily bacterial and fungal keratitis cases, therefore the outcome of the meta-analysis should be interpreted in the context of these types of infection. All RCTs were completed without any significant drop out and there was no concern with the safety of AMT.

The reporting and categorisation of the baseline severity of IK was considerably variable among the RCTs. Two studies included corneal ulcers with a mean diameter of 5-7 mm (severe disease) (Tabatabaei et al., 2017, Zeng et al., 2014), one study included mainly moderate (2-5 mm in diameter) and severe (> 5mm in diameter) ulcers, and one did not report the baseline severity. Such heterogeneity highlights the need for standardised reporting and a core outcome set in the future clinical trials related to IK. Of the three RCTs that reported the baseline severity of IK, the majority of the included participants/eyes were affected by moderate-to-severe IK, suggesting that adjuvant AMT would be useful for this group of patients. Future studies examining

the effectiveness and safety of adjuvant AMT in mild-to-moderate IK would be required before being routinely applied in clinical practice.

Another aspect that was not examined in this systematic review is the effects of different surgical techniques and types of AMT on the clinical outcomes. Depending on the clinical need and circumstances, AMT can be performed using three different surgical techniques; (a) overlay / patch technique: an AM that is larger than the epithelial defect (epithelial side-up or side-down) is transplanted to cover the ocular surface, allowing the host corneal epithelium to regenerate under the AM; (b) inlay / graft technique: an AM of similar size to the epithelial defect is transplanted to act as a substrate for the host epithelium to grow over; and (c) sandwich technique: a combined overlay and inlay technique that facilitates the regeneration of host epithelium between the AMs (Jirsova and Jones, 2017). In addition, AM can be used in fresh form or preserved for longer-term storage purpose using several methods, including cryopreservation, lyophilisation (freeze-drying) and air-drying methods, with comparable clinical efficacy observed amongst these methods (Jirsova and Jones, 2017). While studies have demonstrated the influence of preservation method on the structural and functional properties of AMs, evidence from clinical studies is lacking in the literature. Cryopreserved AMs, depending on the duration of the preservation, have been shown to possess no or less viable cells compared to fresh, freeze-dried or vacuum-dried AM, which have more viable cells with proliferative capability (Jirsova and Jones, 2017, Allen et al., 2013). On the other hand, some studies have demonstrated that lyophilisation of the AMs may lead to a greater reduction in the growth factors compared to cryopreservation. Preclinical studies observed similar efficacy in terms of healing activity between cryopreserved and freeze-dried AMs, supporting the use of both types of AMs in clinical setting (Libera et al., 2008, Nakamura et al., 2004). In this systematic review, the three RCTs included in the meta-analysis for time to complete healing outcome, one RCT employed

cryopreserved AMT, another RCT utilised freeze-dried AMT and the remainder did not report the preservation method used. Therefore, it is not possible to perform a head-to-head comparison between each type of AM due to limited studies, but the overall effect of either type was positive.

While there was a statistically significant shortening of the healing time by 4 days after adjuvant AMT, the benefit of the procedure needs to be balanced by the availability of workforce, cost of the procedure, and healthcare resources for accommodating the surgical procedure, particularly when this procedure is usually performed as an emergency procedure. Considering the current evidence, there may be limited benefit to perform adjuvant AMT during the acute stage of bacterial and fungal keratitis and should be reserved as the second-line treatment for medically refractory cases.

6.4.3. Quality of evidence and potential biases in the review

Similar outcome reporting in the included RCTs has enabled the meta-analysis, which demonstrated the value of adjuvant AMT in expediting complete corneal healing, reducing corneal vascularisation and potentially achieving better visual outcome. However, two RCTs have some concerns on the risk of bias and another two RCTs have high risk of bias. due to the lack of information in the randomisation process, lack of blinding, and under-powered sample size. In addition, none of the RCTs were prospectively registered with any clinical trial database, which limits the assessment of selective reporting of the outcomes.

Of all four RCTs, only Tabatabaei et al. (2017) conducted a sample size calculation based on the anticipated difference in the size of corneal scar (mean effect size of 4mm^2, standard deviation of 6mm^2, and power of 90%), which yielded a sample size of 48 eyes in each group. However, it is noteworthy to mention that size of corneal

scar is not a routine outcome measure for IK. Based on the findings of this systematic review, an appropriate sample size (using time to complete corneal healing as the main outcome measure) is estimated to be at approximately 200 participants / eyes (based on a mean difference or effect size of 4.08 days, standard deviation of 14.56 days, power of 80% and alpha of 5%). This sample size is also supported by some previous RCTs examining the effectiveness of therapeutic CXL (or PACK-CXL) for IK, where an appropriate sample size was estimated at around 200-250 participants, using time to complete corneal healing as the main outcome measure (Hafezi and Kling, 2016, Ting et al., 2019e). This large sample size would then potentially control for various potential confounding variables that could affect the time to complete healing, including the demographic factors, initial size and severity of the corneal ulcer, types of organisms, and underlying comorbidity (e.g. diabetes).

Another important bias, which is a common issue in surgical trial, is related to the difficulty in blinding (or masking) the participants and the surgeons (Gurusamy et al., 2009, Karanicolas et al., 2010). This is evident in this systematic review where none of the RCTs had implemented or reported any measure to ensure the masking of participants or surgeons, which could potentially lead to risk of bias. In a trial, masking can be introduced to 5 groups of individuals, including participants, surgeons, data collector, outcome adjudicators and data analysts. While it is always possible to mask the latter three groups (i.e. data collectors, outcome adjudicators and data analysts), masking of the participants or surgeons may not always be possible, depending on the surgical intervention and design. For instance, in AMT trial, it would have been difficult to mask the patients or surgeons as the patients would have known that an AMT has been performed (unless a sham surgery is performed) and the surgeons would have seen the physical presence of the AM postoperatively. However, masking the other 3 groups of individuals could reduce the bias. As fewer than 10 studies were

eligible for inclusion, it was not possible to use a funnel plot to identify any publication bias.

In conclusion, this systematic review and meta-analysis demonstrated the benefit of early adjuvant AMT in accelerating corneal healing and improving visual outcome in moderate-to-severe bacterial and fungal keratitis. However, further adequately powered and well-designed RCTs are required to ascertain the true potential of adjuvant AMT in treating active IK, particularly herpetic and Acanthamoeba keratitis. Future standardisation of the baseline assessment and core outcome set for clinical trials related to IK would also be invaluable.

CHAPTER 7

Design and Development of Hybrid Human-Derived Host Defense Peptides for Infectious Keratitis: An In Vitro and In Vivo Study

7.1. Introduction

Bacterial keratitis has been shown to be the main cause for infectious keratitis (IK) in many developed countries, including the US and the UK (>90% cases), with *Staphylococci spp.* (30-60%) and *Pseudomonas aeruginosa* (10-25%) being the two most common bacteria reported (Ting et al., 2021j, Ung et al., 2019b, Ting et al., 2021h, Asbell et al., 2020, Tan et al., 2017, Ting et al., 2018). The current management of IK is challenged by several factors, including the low culture yield (Ting et al., 2021j, Ung et al., 2019b), polymicrobial infection (Khoo et al., 2020, Ting et al., 2019c), and emerging trend of antimicrobial resistance (AMR) (Asbell et al., 2015, O'Neill, 2016, Ting et al., 2021j).

Host defense peptides (HDPs) play vital roles in the innate immune system (Hancock and Lehrer, 1998, Mookherjee et al., 2020). Previous studies have demonstrated that numerous HDPs, including LL-37, human beta-defensin (HBD)-2 and -3, are expressed at the OS and upregulated during IK (Mohammed et al., 2017, McIntosh et al., 2005, Haynes et al., 1998, 1999, Mohammed et al., 2020, Otri et al., 2010). HBD-9, on the other hand, was shown to be downregulated during IK (Abedin et al., 2008). In addition, it was demonstrated that the modulated levels of HBD-3 and HBD-9 on OS during bacterial keratitis returned to normal baseline following complete

healing of ulcers (Otri et al., 2012b). Furthermore, HDPs exhibit moderate *in vitro* antimicrobial activity against common ocular surface (OS) pathogenic isolates such as *S. aureus* and *P. aeruginosa* (Huang et al., 2007a, Gordon et al., 2005), highlighting their essential functions in human OS defense.

Despite their promising potential as effective antimicrobial therapies, several issues have impeded the successful translation of HDPs to clinical use. These include their complex structure-activity relationship, susceptibility to host / bacterial proteases, physiological conditions, and toxicity to host tissues, amongst others (Fjell et al., 2011, Li et al., 2017, Ting et al., 2020a). In view of these issues, a number of novel strategies, including residue substitution, chemical modification, and hybridisation, have been proposed to enhance the therapeutic potential of HDPs (Fjell et al., 2011, Li et al., 2017, Ting et al., 2020a).

A number of hybrid peptides, including cecropin A-melittin (Boman et al., 1989), cecropin A-LL37 (Wei et al., 2016), and melittin-protamine (Willcox et al., 2008), amongst others (Ting et al., 2020a), have been previously designed and reported in the literature. When compared to the parent peptide, these hybrid peptides demonstrated improved antimicrobial efficacy and/or reduced toxicity to host tissues. However, strategies in combining two human-derived HDPs from two different classes (e.g. LL-37 and HBD) have not been previously explored. This study aimed to develop novel topical antimicrobial treatment for Gram-positive bacterial keratitis, particularly *S. aureus* (SA) and methicillin-resistant SA (MRSA), using hybridised HDPs derived from different combination of LL-37 and HBD-1 to -3, which are all important HDPs expressed at the OS. The efficacy and safety of the most promising molecule, CaD23 (a hybrid derivative of LL-37 and HBD-2), was further examined and validated in murine corneal wound healing and bacterial keratitis models.

7.2. Materials and methods

The commercially synthesised peptides and commonly used antibiotics for IK were first examined for their *in vitro* antimicrobial efficacy against a range of bacteria. *In vitro* cytotoxicity of these antimicrobial agents was then determined against human corneal epithelial cells (HCE-2, CRL-11135, ATCC, UK) using cell viability assay and cytotoxicity assay, and against human erythrocytes using haemolytic assay. The most promising synthetic peptide, CaD23, was further examined for its time- and concentration-dependent *in vitro* antimicrobial activity. All the assays described in this study were conducted in biological duplicate and in at least two independent experiments, with appropriate positive controls (PCs) and negative controls (NCs). The development pathway of the hybrid HDPs is illustrated in **Figure 7.1**.

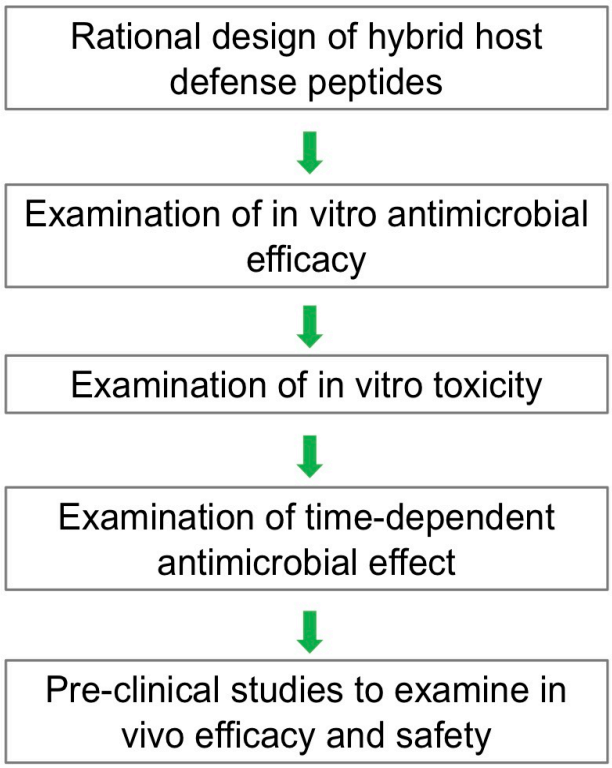


Figure 7.1. The development pathway of hybrid host defense peptides (HDPs).

7.2.1. Design and synthesis of HDPs

A template-based design method was used to design our human-derived HDPs. The native peptide sequences were obtained from an established protein bank database (https://www.uniprot.org/). Several human-derived HDPs, specifically HBD-1, -2, and -3, and cathelicidin (LL-37), were subject to testing and hybridization. The physicochemical properties of the designed peptides, including peptide weight, net charge, hydrophobicity (<H>), and amphiphilicity / hydrophobic moment (<μH>, were analyzed using a computational programmes such as PepCalc (https://pepcalc.com/) and HeliQuest (http://heliquest.ipmc.cnrs.fr). Various strategies, including residue substitution and hybridization, were employed for SAR analysis and for improving the therapeutic index (i.e. increasing the antimicrobial efficacy and reducing the toxicity profile). The native and synthetic peptide sequences are shown in **Table 7.1**.

Table 7.1. Summary of the native and synthetic host defense peptide (HDP) sequences.

Table 7.1. Summary of the native and synthetic host defense peptide (HDP) sequences.

Types	Peptides	Peptide sequence	Number of AA	Molecular weight, g/mol	Net charge	Hydrophobicity, <H>	Hydrophobic moment, <μH>
Native	HBD1	DHYNCVSSGG QCLYSACPIF TKIQGTCYRG KAKCCK	36	3934.6	+4	0.431	0.348
	HBD2	GIGDPVTCLK SGAICHPVFC PRRYKQIGTC GLPGTKCCKK P	41	4334.2	+6	0.489	0.246
	HBD3	GIINTLQKYY CRVRGGRCAV LSCLPKEEQI GKCSTRGRKC CRRKK	45	5161.2	+11	0.228	0.097
	LL37	LLGDFFRKSK EKIGKEFKRI VQRIKDFLRN LVPRTES	37	4493.3	+6	0.201	0.521
Synthetic single HDPs	Ca12	KRIVQRIKDF LR	12	1571.9	+4	0.193	0.782
	BD2*	KCCKKP	6	705.9	+3	-	-
	BD3	RGRKCCRRKK	10	1290.6	+7	-0.393	0.300
1st generation HyHDPs	DD12	RGKAKCCKGT KCCKKP	16	1739.2	+7	0.031	0.144
	DD13	RGKAKCCKRG RKCCRRKK	18	2165.7	+11	-0.251	0.200
	DD32	RGRKCCRRKK KCCKKP	16	1978.5	+10	-0.194	0.201
	CaD1	KRIVQRIKDF LRRGKAK	17	2112.6	+7	-0.022	0.383
	CaD2	KRIVQRIKDF LRKCCKKP	18	2259.8	+7	0.174	0.445
	CaD3	RIKDFLRNGR KCCRRKK	17	2177.7	+8	-0.118	0.234
2nd generation HyHDPs	CaD21	KRIVQRIKDF LRKACKKP	18	2227.8	+7	0.106	0.443
	CaD22	KRIVQRIKDW LRKCCKKP	18	2298.9	+7	0.200	0.456
	CaD23	KRIVQRIKDW LRKLCKKW	18	2398.0	+7	0.294	0.456

HBD = Human beta-defensin; AA = Amino acids; HyHDPs = Hybrid host defense peptides
C = Cysteine; D = Aspartic acid; E = Glutamic acid; F = Phenylalanine; G = Glycine; I = Isoleucine; K = Lysine; L = Leucine; N = Aspargine; P = Proline; Q = Glutamine; R = Arginine; S = Serine; T = Theorine; V = Valine; W = Tryptophan
Calculation of the physicochemical properties of the peptides was performed using PepCalc (https://pepcalc.com/) and HeliQuest (https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py)
*The sequence is too short for calculation of hydrophobicity and hydrophobic moment.

Synthesis of the single and hybrid HDPs was based on the knowledge of the functional regions of the native templates. Three single, short-sequenced (truncated) peptides, based on the native template of HBD2, HBD3 and LL-37, were first generated. Truncated versions of the parent peptides were engineered as this strategy has been shown to serve as a useful method in improving the efficacy of peptides and reducing the cost of synthesis, which represents a significant translational barrier of peptide-based antimicrobial therapy. The C-terminal region of HBD2 and HBD3 was synthesised and examined in view of the presence of high cationicity (i.e. rich of lysine and/or arginine residues), which are important for the antimicrobial efficacy (Hancock and Sahl, 2006, Krishnakumari and Nagaraj, 2012, Dhople et al., 2006). In addition, the middle region of LL-37, same as the KR12 molecule, was synthesized as it has been shown to exhibit efficacy equivalent to the full-length of LL-37 (Wang, 2008b).

All antibiotics were purchased from Sigma-Aldrich, United Kingdom. The peptides were commercially produced by Mimotopes (Mimotopes Pty. Ltd., Mulgrave Victoria, Australia) via traditional solid phase Fmoc synthesis method. All the synthetic peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) to >95% purity and characterised by mass spectrometry. In view of the hydrophobicity, the peptides were first fully dissolved in 50 μl of dimethyl sulfoxide (DMSO) followed by dilution in sterile, de-ionised water to achieve a final concentration of 1 mg/ml peptide in 0.5% DMSO. Further dilution was performed for specific assays as required.

7.2.2. Range of microorganisms being tested

A range of Gram-positive and Gram-negative laboratory- and clinical-strain bacteria were used for the experiments. These included laboratory-strain methicillin-sensitive

S. aureus (MSSA; including SH1000 and ATCC SA29213), laboratory-strain MRSA (ATCC MRSA43300), clinical-strain MRSA, laboratory-strain methicillin-sensitive *Staphylococcus epidermidis* (MSSE; ATCC SE12228), clinical-strain methicillin-resistant SE (MRSE), and laboratory invasive-strain *P. aeruginosa* (PAO1-L).

7.2.3. Determination of *in vitro* antimicrobial efficacy

In vitro antimicrobial efficacy of the antibiotics and designed HDPs was determined using an established minimum inhibitory concentration (MIC) assay with broth microdilution method approved by the Clinical and Laboratory Standards Institute (CLSI) (Clinical & Laboratory Standards Institute (CLSI), 2019). Briefly, the microorganisms were cultured on Tryptone Soya Agar (TSA) and incubated overnight for 18-21 hours at 37 ◦C. Bacterial inoculums were subsequently prepared using the direct colony suspension method (Clinical & Laboratory Standards Institute (CLSI), 2019). Three to five bacterial colonies were obtained from the agar plate and inoculated into an Eppendorf tube containing 1 ml of cation-adjusted Muller-Hinton broth (caMHB), consisting of 20-25 mg/L calcium ions (Ca^{2+}) and 10-12.5 mg/L magnesium ions (Mg^{2+}). The bacterial suspension was adjusted to achieve a turbidity equivalent to 0.1 OD_{600} or 0.5 MacFarland, containing ~1.5 x 10^8 colony-forming unit (CFU)/ml, which was then further diluted in 1:150 in caMHB to reach a final bacterial concentration of ~1x10^6 colony forming units (CFU)/ml. Subsequently, 50 μl of 1x10^6 CFU/ml bacteria and 50 μl of treatment / controls were added into each well for the MIC assay. As the HDPs are known to be influenced by the salt content (Ting et al., 2020a). the MIC assay was also performed in the presence of physiological tear salt concentration (150 mM NaCl). The MIC values, defined as the lowest concentration of the antimicrobial agent that prevented any visible growth of bacteria, were determined after 24 hours of incubation with treatment.

7.2.4. *In vitro* cell viability and cytotoxicity assays

ATCC-authenticated human corneal epithelial cell line (HCE-2, CRL11135, maintained in-house) was used during *in vitro* cell viability and cytotoxicity assays. This cell line was derived from a primary culture of normal human corneal epithelium that was immortalised with adenovirus 12-simion virus 40 (Ad12-SV40) hybrid virus. The cell viability and cytotoxicity of the antibiotics and designed HDPs were determined using commercialised colorimetric assays, including the cell-counting-kit-8 (CCK-8) assay (Sigma Aldrich, Merck Life Science UK Limited, Dorset, UK) and lactate dehydrogenase (LDH) assay (ThermoFisher Scientific, UK), respectively.

CCK-8 assay is a cell viability assay that utilises highly water-soluble tetrazolium salt, WST-8, to examine the intracellular dehydrogenase activity of living cells. The WST-8 is reduced by the dehydrogenase activities in the living cells to yield a yellow-coloured formazan dye (**Figure 7.2**). The amount of change in colour served as a proxy for cell viability and was quantitatively measured by the CLARIOstar plate reader (BMG LABTECH Ltd., Bucks, UK) at absorbance 450 nm. The detection sensitivity of CCK-8 assay has been shown to be more superior than the other cell viability assays that uses other types of tetrazolium salts such as MTT, MTS, XTT, and WST1 (Aslanturk, 2017, Ginouves et al., 2014). Cell viability was calculated using the following formula: $[((I_{treatment} - I_{NC}) / (I_{NC})) \times 100$; I=intensity]

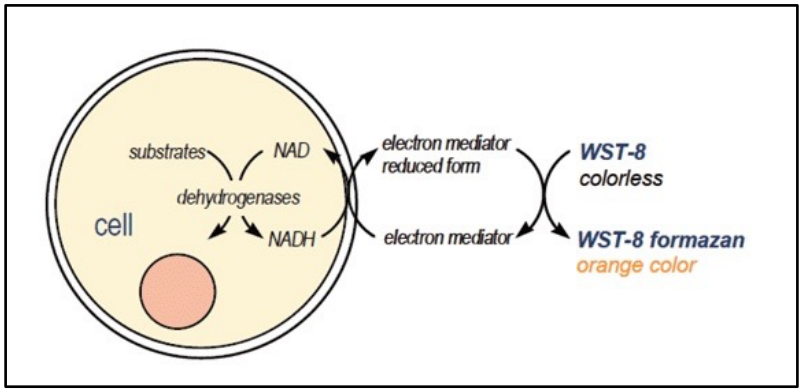


Figure 7.2. The principle of CCK-8 cell viability assay utilising WST-8.

LDH assay is a cytotoxicity assay that measures the extracellular content of LDH. This is based on the principle that LDH is a common cytoplasmic enzyme that is found in almost all living cells. LDH is impermeable to functioning membrane and the extracellular leakage of cytoplasmic LDH indicates the disruption of cell membranes, which is used as a proxy for cytotoxicity. Extracellular LDH catalysed the conversion of lactate to pyruvate through NAD+ reduction to NADH, which allows diaphorase to reduce iodonitrotetrazolium (INT) salt to red formazan, which can be quantitatively measured (**Figure 7.3**). Cytotoxicity was calculated using the following formula: $[((I_{treatment} - I_{NC}) / (I_{PC} - I_{NC})) \times 100]$.

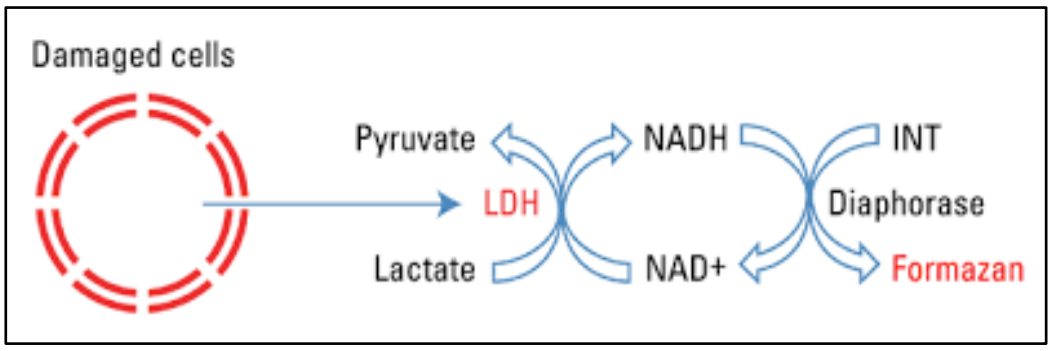


Figure 7.3. The principle of lactate dehydrogenase cytotoxicity assay.

Briefly, HCE-2 cells were cultured and seeded into a 96-well plate at a density of 7.5 x 10^3 cells/well and allowed to attach overnight and grew to 80-90% confluency in keratinocyte serum free medium (KSFM) supplemented with human recombinant epidermal growth factor, bovine pituitary extract, hydrocortisone, and insulin. Once the confluency reached 80-90%, the cells were incubated with treatment for 3 hours before OD measurement was taken using BMG Clariostar microplate reader (BMG Labtech Ltd., Aylesbury, UK). Appropriate controls were used, including 0.1% Triton X-100 as positive control and KSFM as negative control.

7.2.5. Haemolytic assay

Ethical approval was obtained from the Local Research Ethics Committee of University of Nottingham prior to the experiment (Study Reference: 176-1812). Haemolytic assay was performed according to the previously established protocol (Mohammed et al., 2019). Briefly, 5 ml of fresh human blood was collected from healthy participants with informed consent, in an EDTA tube and centrifuged for 10 mins at 1300 g at 10 °C for separation of plasma. The remaining erythrocytes were rinsed and centrifuged further three times in Ca^{2+}/Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS). Subsequently, erythrocytes were diluted to 8% v/v in DPBS and incubated with 100 µl of treatment, positive control (1% Triton X-100) and negative control (DPBS), all in 1:1 ratio, for 1 hour (final erythrocytes concentration = 4% v/v). After 1 hour of incubation, the plate was centrifuged at 500 x g for 5 mins and 100 µl of supernatant of each well was transferred into a 96-well plate for measurement at OD_{540}. Haemolysis (%) was calculated as $[(I_{treatment} - I_{NC}) / (I_{PC} - I_{NC})]$ x 100.

7.2.6. Time-kill kinetics assay

Time-kill kinetics assay was performed to determine the time-dependent and concentration-dependent *in vitro* antimicrobial effects of the peptide (0.25x and 2x MIC) and amikacin, a commonly used topical antibiotics for bacterial keratitis (8x and 20x MIC), against 100 μl of ~1 x 10^6 CFU/ml of SH1000 at various time points, including 0 min (pre-treatment), 15 mins, 30 mins, 60 mins, 2 hours, 4 hours, and 24 hours. At each time point, 10 μl of the treated bacteria was transferred to an Eppendorf tube containing 90 μl of PBS, which was then serially diluted in 1:10 concentration for inoculation on agar plates and incubated overnight for 18-21 hours at 37 °C for enumeration of CFU.

7.2.7. In vivo efficacy and safety studies

The in vivo studies were conducted in two stages, namely the corneal wound healing study (for safety) and bacterial keratitis study (for efficacy), based on previously established protocols (Lin et al., 2017, Mayandi et al., 2020). Wild-type C57BL/6J mice (8-9 weeks old, male, average weight of 25g) were used in view of the consistent and reproducible results demonstrated in previous studies (Lin et al., 2017, Mayandi et al., 2020). The mice were maintained and treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council) and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All animal studies were conducted at the Singapore Eye Research Institute, Singapore, and were approved by the Animal Welfare & Ethical Review Body (AWERB), University of Nottingham, UK (Ref: UoN-Non-UK #16), the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) of SingHealth, Singapore (Study Reference: 2019/SHS/1491). General anaesthesia was administered using intraperitoneal injections of xylazine (10 mg/kg) and ketamine (80 mg/kg)], and a drop of topical proxymetacaine hydrochloride 0.5% was administered

immediately before wounding and/or infecting the corneas. Upon completion of the experiments, all mice were sacrificed according to the method of humane killing set out in Animals (Scientific Procedures) Act 1986 Schedule 1 using overdose of general anaesthesia via intraperitoneal route.

7.2.7.1. In vivo corneal wound healing study

Drug drainage, blinking and tear film were considered during dosing translation from *in vitro* to *in vivo* use.[51] Based on the MIC of CaD23 against *S. aureus* ATCC 29213 (= 25 μg/ml), a range of concentration of CaD23 was chosen, including 300 μg/ml (0.03%; 12x MIC), 500 μg/ml (0.05%; 20x MIC) and 1mg/ml (0.1%; 40x MIC).

In vivo safety of CaD23 (in 0.03%, 0.05% or 0.1%), and PBS (negative control) was first determined in a mouse corneal epithelial wound healing model. In the absence of *in vivo* pilot data of our designed HDPs, the sample size was calculated based on a previous study (Aung et al., 2016). This was designed as a non-inferiority trial to ensure that the HDPs did not affect the wound healing when compared to PBS (control). The non-inferiority margin was set at 10% difference of the wound size between HDPs and PBS at 3 days (deemed as significantly different), with a standard deviation (SD) of 5% (Cohen's d=2.0), power=80% and $p<0.05$. A minimum sample size of 4 mice / treatment group was needed.

All mice were randomly allocated to each of the four treatment groups (n = 4 mice / group). Prior to the study, all eyes were examined with slit-lamp biomicroscopy to confirm the health of corneas. Under general and topical anaesthesia, the central 2 mm corneal epithelium was gently debrided with sterile Beaver mini-blades, leaving the basal lamina intact. Each treatment was applied immediately after wounding, then 4 times a day at 3-hour interval for 3 days (total dose of treatment per mouse = 14). Corneal epithelial defect was assessed using a cobalt-blue filter-equipped slit-lamp

biomicroscopy and photography with staining with topical sodium fluorescein 1% at baseline (immediately post-debridement) and daily up to 3 days post-treatment. Fluorescein-stained images of the corneal wound defect were analysed using ImageJ software. The main outcome measure was the wound size at the end of day 1, 2 and 3 [expressed as % of the original wound size in mean and standard deviation (SD)]. Difference in the wound size between groups was analysed using one-way ANOVA with Dunnett's post hoc test (PBS as the control group).

7.2.7.2. In vivo *S. aureus* keratitis study

Based on the in vivo safety data, the highest tolerable concentration, 0.05%, of CaD23 was used in the subsequent *S. aureus* keratitis murine model. Levofloxacin 0.5% (a commonly used antibiotic for bacterial keratitis in clinical setting) and PBS were used as the positive and negative controls, respectively. In the absence of *in vivo* pilot data, the sample size was calculated based on a previous study (Lin et al., 2017). To detect an effect size of 1 LogCFU (or 10 times) difference in the bacterial load (significant antimicrobial efficacy) between HDPs (mean=5 logCFU) and PBS (mean=6 logCFU; NC), with a SD of 0.5 logCFU (Cohen's d=2.0), power=80% and $p<0.05$, a minimum sample size of 4-5 mice/group is required.

All mice were randomly allocated to each treatment group (n = 5 mice / group). Slit-lamp examination was performed before the start of experiment to confirm the health of corneas. Under general and topical anaesthesia, the central 2 mm corneal epithelium was gently removed with sterile Beaver mini-blades. 10 μl of ~$1x10^8$ CFU/ml of ATCC SA29213 was applied topically onto the cornea and the lid was held shut for 1 min. At 6 hours post-infection, 10 μl of treatment was applied directly onto the infected corneas with a dose regimen of 4 times a day at 3-hour interval for 3 days (total dose of treatment per mouse = 12). The eyes were monitored daily using slit-lamp biomicroscopy and photography. At the end of day 3, all animals were sacrificed,

and infected eyes were enucleated. The whole corneas were subsequently dissected and homogenised in 1 ml of sterile PBS using sterile glass micro-beads. The homogenised infected corneal tissue suspension was serially diluted in 1:10 and plated on TSA plates in triplicates for enumeration of CFU after 24 hours incubation at 37 °C. The main outcome measure was the residual bacterial load at 3-day post-treatment (expressed as $\log_{10}$ CFU/ml, which was the same as $\log_{10}$ CFU/cornea) and the difference between groups was analysed using one-way ANOVA with Dunnett's post hoc test (PBS as the control group).

7.3. Results

7.3.1. *In vitro* antimicrobial efficacy of HDPs

A total of 12 synthetic HDPs were rationally designed and synthesised based on the templates of native LL-37 and HBD-1 to -3 (**Table 7.1**). The MIC values (in μg/ml and μM) of all the tested antibiotics, single and hybrid peptides, in the absence and presence of 150 mM NaCl are summarised in **Table 7.2**. All 3 single linear, short-sequenced (6-12 amino acids) peptides (based on HBD-2, HBD-3 and LL-37) and 6 first-generation hybrid HDPs (based on different combinations of LL-37, and HBD-1 to -3) did not demonstrate any significant antimicrobial efficacy (MIC>200 μg/ml) against Gram-positive and Gram-negative bacteria. Further 3 second-generation peptides (derived from CaD2 sequence) were engineered through rational modification: (1) CaD21: substitution of cysteine with alanine; (2) CaD22: substitution of phenylalanine with tryptophan (to increase hydrophobicity); and (3) CaD23: substitution of phenylalanine and proline with tryptophan (to further enhance hydrophobicity) and substitution of cysteine with leucine. CaD21 and CaD22 demonstrated slight improvement in the antimicrobial efficacy whereas CaD23 exhibited good antimicrobial efficacy against MSSA (MIC=12.5-25.0 μg/ml), MRSA (MIC=25 μg/ml), MSSE (MIC=12.5 μg/ml) and MRSE (MIC=3.1 μg/ml), highlighting

the importance of increased hydrophobicity against Gram-positive bacteria (**Table 7.1**). Moderate efficacy of CaD23 was observed against *P. aeruginosa* (MIC=50 μg/ml). When tested in the presence of physiological tear salt concentration, the MIC of CaD23 against MSSA, MRSA and MRSE increased by 2- to 4-fold, and remained unchanged for MSSE and *P aeruginosa*.

Table 7.2. Summary of MIC of antibiotics and synthetic HDPs.

Table 7.2. A summary of the minimum inhibitory concentrations (MICs) of various antibiotics and synthetic human-derived hybrid host defense peptides. All experiments were performed in full-strength cationic Muller-Hinton broth (i.e. MHB-2) and in the absence or presence of physiological tear salt concentration (150mM NaCl). The MIC values are presented in μg/ml (μM). When the MIC level was >200 μg/ml in the absence of salt, the peptide was not subjected to testing in the presence of salt.

Class of agent	Agents	SH1000		SA29213		MRSA		MRSA43300		SE12228		MRSE		PAO1-L	
	μg/ml (μM)	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*	*0mM*	*150mM*
Antibiotics	Amikacin	1.25 (2.13)	10 (17.1)	-	-	10 (17.1)	10 (17.1)	-	-	-	-	1.25 (2.13)	5 (8.53)	0.63 (1.08)	1.25 (2.13)
	Levofloxacin	0.31 (0.86)	0.31 (0.86)	-	-	0.31 (0.86)	0.31 (0.86)	-	-	-	-	0.31 (0.86)	0.31 (0.86)	0.31 (0.86)	0.31 (0.86)
First-generation hybrid HDPs	DD12	>200 (>115)	-	-	-	>200 (>115)	-	-	-	-	-	>200 (>115)	-	>200 (>115)	-
	DD13	>200 (>92.3)	-	-	-	>200 (>92.3)	-	-	-	-	-	>200 (>92.3)	-	>200 (>92.3)	-
	D32	>200 (>101)	-	-	-	>200 (>101)	-	-	-	-	-	>200 (>101)	-	>200 (>101)	-
	CaD1	>200 (>94.7)	-	-	-	>200 (>94.7)	-	-	-	-	-	>200 (>94.7)	-	>200 (>94.7)	-
	CaD2	>200 (>88.5)	-	-	-	>200 (>88.5)	-	-	-	-	-	>200 (>88.5)	-	>200 (>88.5)	-
	CaD3	>200 (>91.8)	-	-	-	>200 (>91.8)	-	-	-	-	-	>200 (>91.8)	-	>200 (>91.8)	-
Second-generation hybrid HDPs	CaD21	200 (89.8)	>200 (>89.8)	-	-	>200 (>89.8)	-	-	-	-	-	50 (22.4)	100 (44.9)	100 (44.9)	50 (22.4)
	CaD22	100 (43.5)	>200 (87.0)	-	-	>200 (>87.0)	-	-	-	-	-	6.3 (2.7)	25 (10.9)	200 (87.0)	200 (87.0)
	CaD23*	12.5 (5.2)	50 (20.8)	25 (10.4)	50 (20.8)	25 (10.4)	100 (41.7)	25 (10.4)	100 (41.7)	12.5 (5.2)	12.5 (5.2)	3.1 (1.3)	6.3 (2.6)	50 (20.8)	50 (20.8)

MIC refers to the lowest concentration of antibiotic / peptide that prevents any visible bacterial growth after 24 hours of incubation with treatment. Data represent the mean of two biological duplicate from two to three independent experiments.

*The hybrid HDP that demonstrated good antimicrobial efficacy against Gram-positive bacteria and moderate efficacy against Gram-negative bacteria.

7.3.2. *In vitro* cell viability and cytotoxicity

Amikacin and CaD2 did not demonstrate any lethal effect on HCE-2 cell viability at 200 μg/ml (**Figure 7.4A**). The IC_{50} (concentration that inhibits 50% of the cell viability) of CaD21, CaD22 and CaD23 were >200 μg/ml, >200 μg/ml and 54.6 ± 11.7 μg/ml, respectively. This demonstrated that the increased hydrophobicity with tryptophan residues enhanced the antimicrobial efficacy of CaD23 but with increased negative effect on the cell viability of HCECs. In terms of cytotoxicity, amikacin and CaD2 did not show any sign of toxicity for HCECs at 200μg/ml and CaD23 showed 30.4 ± 7.8% cytotoxicity at 200 μg/ml (**Figure 7.4B**).

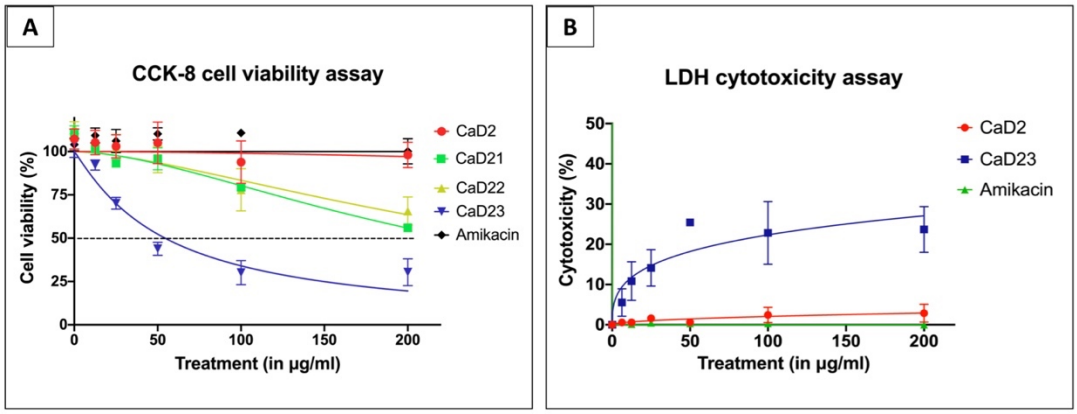


Figure 7.4. Cytotoxicity of HDPs and antibiotic.

Cytotoxicity of synthetic peptides and amikacin (a commonly used antibiotic for bacterial keratitis) in various concentrations against human corneal epithelial cells (HCE-2), presented as dose-response curves (normalized, variable slope). Percentage cell viability is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate. Some error bars are missing due to small standard deviation values. **(A)** Cell viability assay (using cell counting kit-8 assay) demonstrating normal metabolic activity of epithelial cells in CaD2 and amikacin but reduced activity in CaD23 (IC_{50} = 54.6 ± 11.7 μg/ml) after 3 h of treatment. IC_{50} (concentration of treatment inhibiting 50% of cell viability) is shown in a black dotted line. **(B)** Cytotoxicity assay (using lactate dehydrogenase assay) demonstrating no sign of cytotoxicity of epithelial cells in amikacin and CaD2, and low level of cytotoxicity in CaD23 (30.4 ± 7.8% at 200 μg/ml; LC_{50} > 200 μg/ml) after 3 h of treatment.

A summary of the cell viability, cytotoxicity and haemolytic results of CaD23 is provided in **Table 7.3**.

Table 7.3. Summary of toxicity of antibiotics and synthetic HDPs.

Summary of the cytotoxicity, cell viability and hemolytic results of antibiotics and synthetic peptides (in µg/ml concentration). The cytotoxicity and cell viability results were obtained after 3 hours of treatment whereas hemolytic effect of treatment was examined after 1 hour of treatment.

Types	**Agents**	**LC_{50}**	**L_{max} (%)**	**IC_{50}**	**I_{max} (%)**	**HC_{50}**	**H_{max} (%)**
Antibiotics	Amikacin	>200	0.0 (0.1)	>200	100.1 (7.3)	>200	0.0
	Levofloxacin	>200	1.2 (0.7)	>200	108.0 (2.8)	>200	0 (0.0)
First-generation peptides	DD12	-	-	-	-	-	-
	DD13	-	-	-	-	-	-
	DD32	-	-	-	-	-	-
	CaD1	-	-	-	-	-	-
	CaD2	>200	2.9 (2.2)	>200	98.1 (7.3)	-	-
	CaD3	-	-	-	-	-	-
Second-generation peptides	CaD21	-	-	>200	56.1 (1.0)	-	-
	CaD22	-	-	>200	65.7 (8.2)	-	-
	CaD23	>200	26.6 (6.5)	54.6 (11.7)	69.6 (7.8)	>200	7.1 (3.0)

LC_{50} = Concentration of treatment causing 50% cytotoxicity; L_{max} (%) = Percentage of cytotoxicity at 200 µg/ml treatment concentration; IC_{50} = Concentration of treatment causing 50% inhibition of cell viability; I_{max} (%) = Percentage of inhibition of cell viability at 200 µg/ml treatment concentration; HC_{50} = Concentration of treatment causing 50% haemolysis; H_{max} (%) = Percentage of haemolysis at 200 µg/ml treatment concentration

Results are presented in mean (SD) of two independent experiments performed in biological duplicate. Toxicity results of some peptides were missing because their antimicrobial efficacy was poor and hence toxicity was not determined.

Haemolytic assay demonstrated minimal (7.1 ± 3.0%) haemolytic activity of CaD23 at 200 μg/ml (**Figure 7.5**), suggesting that it is potentially safe for systemic use at this concentration.

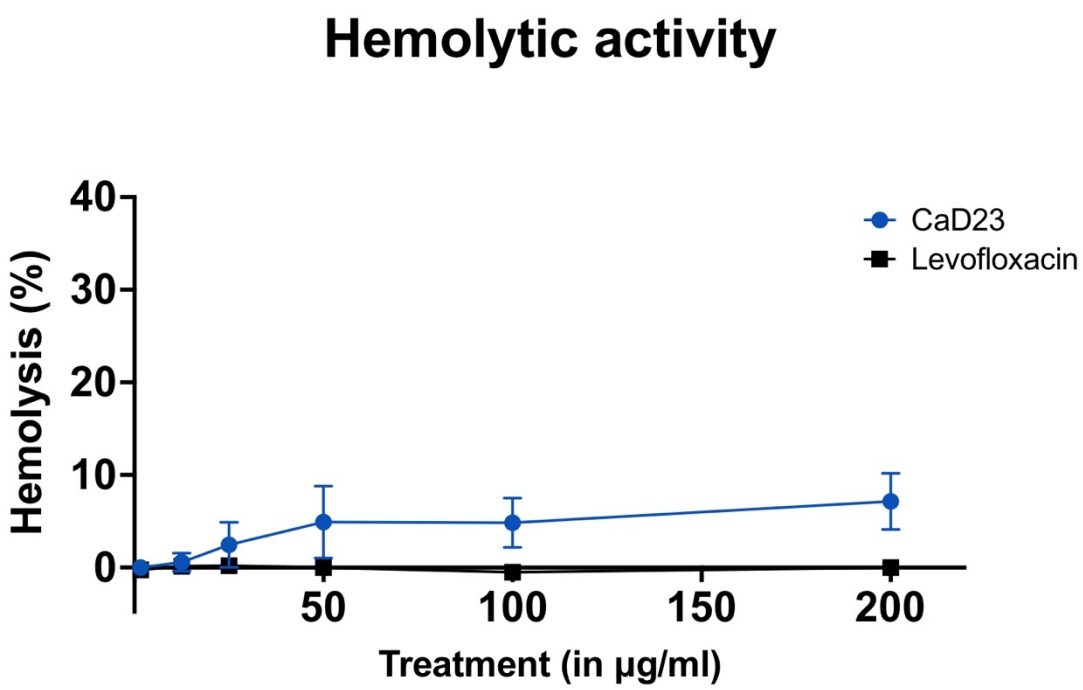


Figure 7.5. Hemolytic effect of CaD23 and levofloxacin.

Haemolytic effect of CaD23 and levofloxacin (a commonly used antibiotic for bacterial keratitis) in various concentrations against fresh human erythrocytes, determined after 1 hour of treatment. Percentage hemolysis is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate. Some error bars are missing due to small standard deviation values. The graph demonstrating minimal hemolytic effect of CaD23 against fresh human erythrocytes (only 7.1 ± 3.0% at 200 μg/ml).

In addition, a summary of the therapeutic index of CaD23 (defined as IC_{50}, LC_{50} or HC_{50} divided by the MIC value) is provided in **Table 7.4**. The LC_{50} (concentration that kills 50% of the cells) of CaD23 was >200μg/ml, which yielded a therapeutic index (defined by LC_{50} divided by the MIC value) of >8 for treating *S. aureus* ATCC29213, and >4 for treating *P. aeruginosa* PAO1-L.

Table 7.4. Summary of therapeutic index of CaD23.

μg/ml	***S. aureus* SH1000**		***S. aureus* ATCC29213**		**Clinical MRSA**		**MRSA ATCC43300**		***P. aeruginosa* PA01-L**	
	MIC	**TI**	**MIC**	**TI**	**MIC**	**TI**	**MIC**	**TI**	**MIC**	**TI**
Based on LC50 (= >200)	12.5	>16	25	>8	25	>8	50	>4	50	>4
Based on IC50 (= 55)	12.5	4.4	25	2.2	25	2.2	50	1.1	50	>1.1
Based on HC50 (= >200)	12.5	>16	25	>8	25	>8	50	>4	50	>4

LC_{50} = Concentration of treatment causing 50% cytotoxicity; IC_{50} = Concentration of treatment causing 50% inhibition of cell viability; HC_{50} = Concentration of treatment causing 50% haemolysis

MRSA = Methicillin-resistant *Staphylococcus aureus*

The minimum inhibitory concentration (MIC) values and therapeutic index (TI) values are presented in mean values.

7.3.3. Time-kill kinetics

Time- and concentration-dependent antimicrobial activity of CaD23 was determined against MSSA (SH1000) using time-kill kinetics assay. When CaD23 was used at 2x MIC (25 μg/ml) against SH1000, it was able to achieve significant killing (99.9% or 3 $\log_{10}$ CFU/ml reduction) with 15 mins and complete killing (100%) within 30 mins of treatment (**Figure 7.6**). In contrast, amikacin (used at 20x MIC; 25 μg/ml) could only achieve significant and/or complete killing (99.9-100%) of SH1000 within 4 hours of treatment, which was 8 times slower than CaD23. The antimicrobial efficacy of CaD23 (25 μg/ml) and amikacin (both 10 μg/ml and 25 μg/ml) were maintained at 24 hours' time-point, with no evidence of bacterial re-growth.

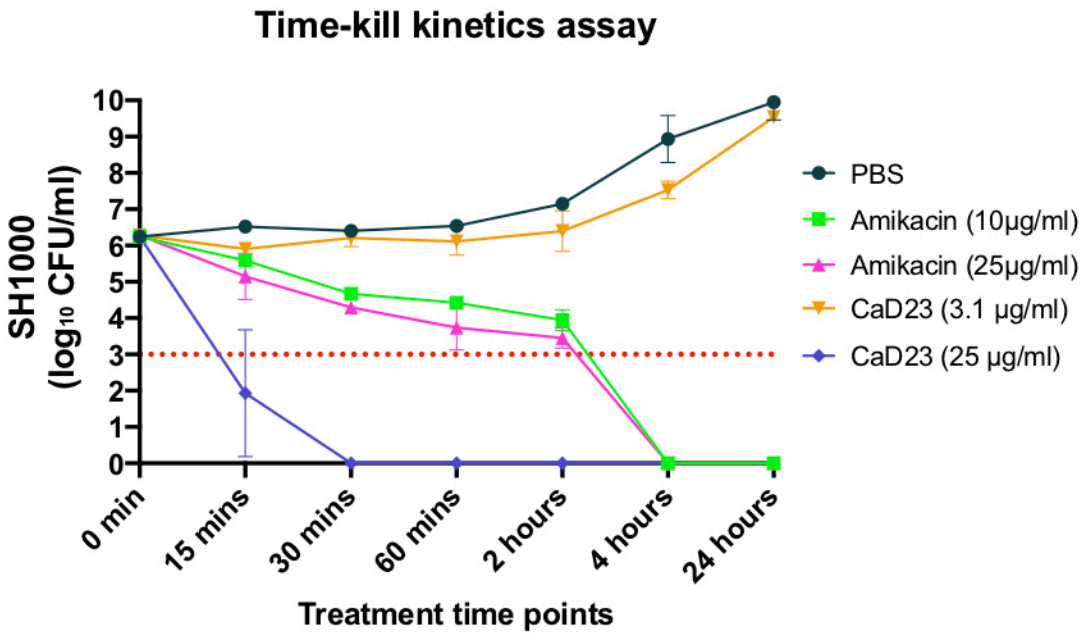


Figure 7.6. Time-kill kinetics assay of CaD23 and amikacin.

Time-kill kinetics of CaD23 [at 3.1 μg/ml (or 0.25x MIC) and 25 μg/ml (or 2x MIC)] and amikacin [at 10 μg/ml (or 8x MIC) and 25 μg/ml (or 20x MIC)] against *S. aureus* (SH1000) over 24 hours. Phosphate buffer saline (PBS) group serves as the untreated control. "0 min" represents the starting inoculum, which is around 6 $\log_{10}$ CFU/ml. The red dotted horizontal line at 3 $\log_{10}$ CFU/ml signifies the threshold of significant bacterial killing (defined as 99.9% or 3 $\log_{10}$ CFU/ml reduction of the bacterial viability compared to the starting inoculum). Data is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate. CaD23 [at 25 μg/ml (or 2x MIC)) was able to achieve complete (100%) killing of SH1000 within 30 mins of treatment whereas amikacin [at 10 μg/ml (or 8x MIC) and 25 μg/ml (or 20x MIC)] was only able to achieve complete killing of SH1000 within 4 hours of treatment. The antimicrobial efficacy of CaD23 and amikacin was maintained at 24 hours post-treatment.

7.3.4. *In vivo* safety of CaD23

In view of the known discrepancy between *in vitro* and *in vivo* results, the safety of CaD23 was further determined in a murine corneal epithelial wound healing model. When compared to the PBS group, both CaD23 0.03% (or 300 μg/ml) and CaD23 0.05% (or 500 μg/ml) groups did not show any significant difference in the rate of corneal re-epithelialisation (all healed within 2-3 days), suggesting that both concentrations were safe for topical application (**Figure 7.7**). However, significant delay in corneal re-epithelialisation was observed in the CaD23 0.1% group, with a mean wound size of 28.5 ± 19.9% at day 3 post-injury (p=0.004).

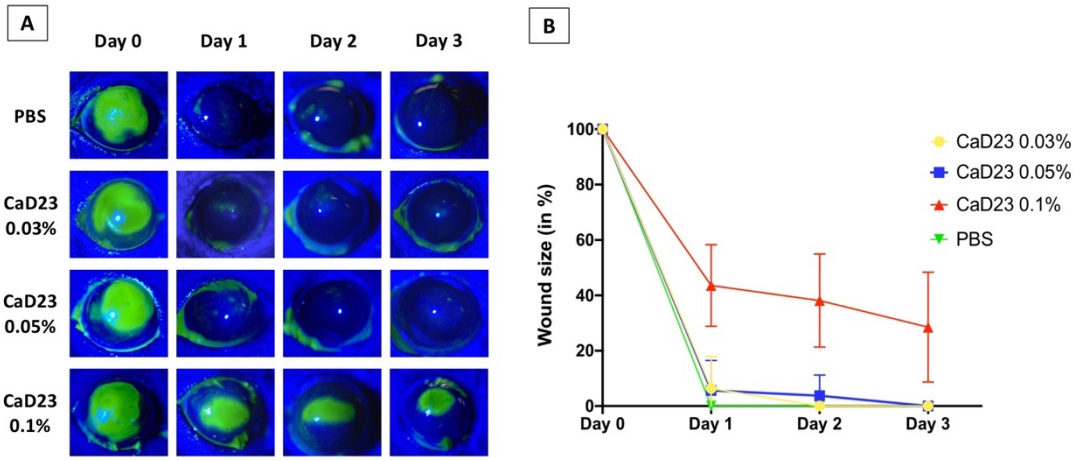


Figure 7.7. In vivo safety of CaD23.

CaD23 in various concentrations [0.03% (or 300 μg/ml), 0.05% (or 500 μg/ml), and 0.1% (1 mg/ml)] and phosphate buffer saline (PBS) assessed in a murine corneal epithelial wound healing model (n = 4 mice / treatment group). **(A)** Representative slit-lamp images showing the daily progress of corneal wound healing of each treatment group. The green color-stained area depicts the corneal epithelial defect. Complete corneal re-epithelialisation was observed in all treatment groups, except CaD23 0.1% group, by day 3. **(B)** Graphical summary of the progress of corneal re-epithelialisation of each treatment group over 3 days. The corneal epithelial wound size at various time points is calculated based on the original 100% wound size at baseline. Data is presented as mean ± standard deviation.

7.3.5. In vivo efficacy of CaD23

Based on the in vivo safety results, the highest tolerable concentration of CaD23 0.05% was subjected to subsequent in vivo efficacy testing in a murine *S. aureus* ATCC 29213 keratitis model. As the data was not normally distributed and was log-transformed, the results were reported in median ± interquartile range (IQR). When compared to the *S. aureus* bacterial viability in the PBS group (4.2 ± 1.3 log_{10} CFU/ml), there was a considerable reduction of bacterial viability in the CaD23 0.05% and levofloxacin 0.5% groups by 94% (3.0 ± 2.4 log_{10} CFU/ml; p=0.72) and 98% (2.4 ± 2.2 log_{10} CFU/ml; p=0.08), respectively, though statistical significance was not achieved (**Figure 7.8**).

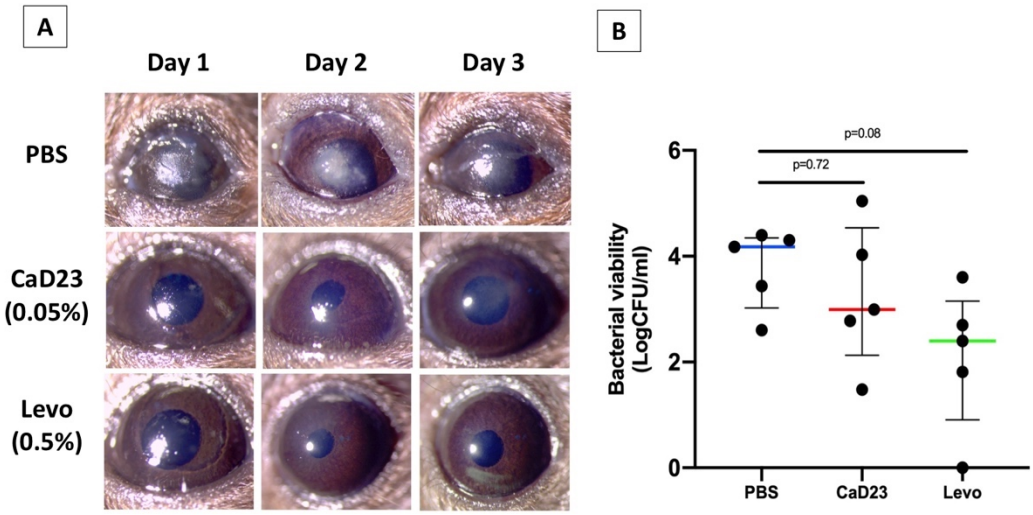


Figure 7.8. In vivo efficacy of CaD23.

In vivo efficacy of CaD23 0.05% (500 μg/ml), levofloxacin 0.5% (positive control) and phosphate buffer saline (PBS; negative control) in a murine *S. aureus* ATCC SA29213 bacterial keratitis model (n = 5 mice / treatment group). **(A)** Representative slit-lamp images showing the corneal appearance over 3 days post-infection in each treatment group. Note the significant infiltrative changes of cornea in the PBS group as compared to the CaD23 0.05% and levofloxacin 0.5% groups. **(B)** Scatter plot showing the bacterial viability of *S. aureus* (in log_{10} CFU/ml) after 3 days of treatment. In view of the wide range of results, data is presented as median ± interquartile range.

7.4. Discussion

IK represents a persistent and uncurbed burden on human health at a global level. Topical antibiotics are the current mainstay of treatment for bacterial keratitis but the efficacy is being increasingly challenged by the emergence of antimicrobial resistance (Ung et al., 2019b, Asbell et al., 2015). In addition, patients with bacterial keratitis often require long duration of treatment and sight-threatening complications may still ensue despite timely intervention (Khor et al., 2018), highlighting an unmet need for newer and better treatment. HDPs serve as an attractive class of antibiotics for treating IK based on the following reasons: (1) the broad-spectrum activity of HDPs can provide comprehensive coverage to a wide range of microorganisms, particularly when mixed infection is relatively common (5-20%) in IK (Ung et al., 2019b); (2) the rapid antimicrobial action help reduce the microbial load and limit the damage to the cornea more effectively, ultimately have a better chance of preserving the vision, as well as reducing the risk of developing antimicrobial resistance, which is currently an emerging issue in ocular infection (Asbell et al., 2015); and (3) HDPs can be used as synergistic or additive agents to the current conventional antibiotics, enhancing the therapeutic index by increasing the antimicrobial efficacy and reducing the dose-related toxicity (Mohammed et al., 2019, Kampshoff et al., 2019).

This study highlights a body of work in designing and developing human-derived hybrid HDP as topical treatment for bacterial keratitis. Hybridisation strategy has been previously employed by several research groups to improve the therapeutic index of HDPs (Wei et al., 2016, Saugar et al., 2006, Willcox et al., 2008) but the strategy of using human-derived hybrid HDPs (with LL-37 and HBD-2) is first of its kind. The initial concept of developing synthetic HDPs derived from LL-37 and HBD was founded on the observation of the upregulation of these key HDPs at the ocular surface during IK (McIntosh et al., 2005, Mohammed et al., 2017, McDermott, 2009,

Otri et al., 2012b). Furthermore, LL-37 and HBD-2 and -3 were shown to exhibit good antimicrobial efficacy against a range of organisms (Huang et al., 2007a, McDermott, 2009). The mid-region of LL-37, consisting of residues 18-29 of LL-37 (i.e. KR-12) was selected as part of the hybrid template as studies have demonstrated that KR-12, though much shorter than parent LL-37, exhibited similar antimicrobial activity against *E. coli* (MIC = ~64 μg/ml) with reduced toxicity to host cells (Wang, 2008b). C-terminal of HBD1-3 was used as the other part of the hybrid template in view of its rich content of cationic residues, which have been shown to play an important role in interacting with anionic bacterial membrane and killing of bacteria (Krishnakumari and Nagaraj, 2012, Dhople et al., 2006).

The initial attempt of engineering single linear HDPs (including LL-37 and HBD-2 and -3) and hybrid HDPs (based on LL-37 and HBD-1 to -3) did not yield any compound with good antimicrobial efficacy. However, with systematic SAR analysis and modification, particularly through substitution of proline and phenylalanine with tryptophan residues (to increase hydrophobicity and membrane partitioning), ensued the development of peptide with enhanced antimicrobial efficacy against MSSA (MIC=12.5μg/ml) and MRSA (MIC=25μg/ml), serving as a proof-of-concept of this novel design strategy. Interestingly, increased hydrophobicity augmented the antimicrobial activity of HDP against Gram-positive bacteria but not Gram-negative bacteria. This is likely attributed to the different compositions in the bacterial membrane between Gram-positive bacteria, which consist of a thick peptidoglycan layer, and Gram-negative bacteria, which comprises an additional outer membrane with abundance of negatively charged lipopolysaccharide for which cationicity of the peptide plays a more important role (Strahl and Errington, 2017). In addition, the efficacy of CaD23 (18 amino acids in length) is at least equal to or stronger than the full-length parent LL37 (MIC = 25-50 μg/ml) and HBD-1 to -3 peptides (MIC = 50-100 μg/ml) (Huang et al., 2007a, Mohammed et al., 2019). Peptides with shorter sequence

not only have the advantage of lower manufacturing cost but may also have a lower risk of inducing immunogenicity (Mahlapuu et al., 2016). Moreover, the antimicrobial efficacy of CaD23 is comparable to some of the HDPs that are developed for ocular surface infection, including esculentin-1a(1-21)NH2 (Kolar et al., 2015), RP444 (Clemens et al., 2017), melimine and its derivatives (Dutta et al., 2017, Willcox et al., 2008), and ε-lysylated Mel-4 (Mayandi et al., 2020).

In the time-kill kinetics study, CaD23 at 2x MIC was able to achieve complete killing of *S. aureus* within 30 mins as compared to 4 hours with amikacin at 20x MIC (i.e., 8 times slower than CaD23). Interestingly, the increase in the concentration of amikacin from 8x MIC to 20x MIC did not expedite its anti-bacterial action against *S. aureus*, suggesting that the speed of bacterial killing is more related to the underlying mechanism of action than the concentration of antibiotic. The rapid killing action of CaD23 is likely related to a membrane perturbation effect, which is in contrast to amikacin where it exerts its anti-bacterial activity via intracellular inhibition of the 30S subunit of bacterial ribosome (Kapoor et al., 2017). Theoretically, the rapid and membrane disruptive action of CaD23 should result in a lower risk of developing antimicrobial resistance as the bacteria has less time to adapt and require substantial modification of genome to develop effective resistant mechanisms, which are shown in conventional antibiotics (Mayandi et al., 2020). Further studies examining the underlying mechanism of action of CaD23 and its tendency to develop AMR would be valuable.

Based on the LDH cytotoxicity assay, CaD23 was shown to be relatively non-cytotoxic (20-30% toxicity) at 200 μg/ml, with a therapeutic index of >8-16 against *S. aureus* (defined by LC_{50} concentration divided by MIC value). In addition, the haemolytic activity of CaD23 at 200 μg/ml was only less than 10%. Interestingly, the IC_{50} value

(based on cell viability assay) was around 50 μg/ml, considerably lower than the LC_{50} value. This discrepancy may be due to the fact that some of the cells were apoptotic instead of necrotic, which was detected by the cell viability assay but not the cytotoxicity assay. That said, the in vivo corneal epithelial wound healing study showed that CaD23 did not demonstrate any significant toxicity when used at 500 μg/ml (or 0.05%), which was 10 times higher than the IC_{50} value. This suggests that *in vitro* plate-based static assays such as LDH or cell viability assays could potentially overestimate the in vivo cytotoxicity of drugs that are developed for ocular topical application. However, there were some signs of delayed corneal wound healing in the CaD23 0.01% group (20 times the IC_{50} value), suggesting that this concentration has limited therapeutic potential due to the cytotoxicity. The experiment was terminated at day 3 post-treatment, which was the pre-specified endpoint of the study; therefore, the complete healing time in the CaD23 0.01% could not be determined. However, the delayed healing in this group at day 3 had provided sufficient and important information on the *in vivo* cytotoxicity of CaD23.

The observed discrepancy between *in vitro* and *in vivo* findings is likely attributed to the inherent dynamic environment of ocular surface with eye blinking, high tears turnover, and drainage (Farkouh et al., 2016). The shortcoming of *in vitro* assays may be addressed by the recently developed novel ex vivo biomimetic model where it could simulate the dynamic and complex interface between the ocular surface and the external environment, allowing a better prediction of the *in vivo* effect (Seo et al., 2019).

The *in vivo* bacterial keratitis study demonstrated that CaD23 0.05% (20x MIC) was able to reduce the bacterial viability of *S. aureus* by 94% when compared to the untreated control group, which was more than the endpoint (i.e. 1 logCFU or 90% reduction in the bacterial bioburden) used in this study. However, the effect was not

statistically significant due to the considerably variable standard deviation observed in both the treatment and the control groups. This similarly explained the insignificant improvement in the levofloxacin-treated group, though there was 1.8 logCFU median reduction in the bacterial bioburden compared to the PBS group. Nevertheless, these results serve as a strong proof-of-concept that CaD23 may be employed as a potentially efficacious treatment for treating Gram-positive bacterial keratitis, but a larger sample size will be required to fully ascertain its efficacy. In addition, we had chosen to sacrifice the mice at day-3 post-infection because *S. aureus* keratitis had been shown to be most severe in C57BL/6J mice at 3-day post-infection, as compared to other stains of mice such as BALB/c and A/J mice, though the infection spontaneously improves at day-5 post-infection and beyond in C57BL/6J mice (Girgis et al., 2003). To investigate the longer-term in vivo antimicrobial efficacy of CaD23 in *S. aureus* keratitis, other strains of mice may be required to examine this aspect.

One potential approach to improve the therapeutic effect of HDP-derived antimicrobial treatment is to use them in combination with antibiotics as peptide-antibiotic synergism has been demonstrated in several studies (Lakshminarayanan et al., 2016, Kampshoff et al., 2019, Mohammed et al., 2019). This attractive antimicrobial strategy not only helps extend the lifespan and broaden the antibacterial spectrum of conventional antibiotics, but also reduce the dose-dependent toxicity associated with HDPs and antibiotics (Mishra et al., 2017). Recently, Mohammed et al. (2019) has also shown that FK16, a cathelicidin-derived molecule, could improve the antimicrobial efficacy of vancomycin, a glycopeptide antibiotic, against PA by 8-fold. This is likely ascribed to the different mechanisms of action between FK16 and vancomycin where the membrane disruptive action of FK16 facilitates the diffusion of vancomycin across the bacterial membrane. Hence, future work investigating the

potential synergism between CaD23 and commonly used antibiotics for bacterial keratitis would be useful.

At present, the aim is to develop the human-derived hybrid HDPs as topical treatment for bacterial keratitis. So far, there are only a few HDP-based treatment that have been developed for treating and/or preventing IK. Comparing with the developed molecules, including Mel4 (which had advanced into phase 3 randomised clinical trial),(Kalaiselvan et al., 2021, Willcox et al., 2020, Kolar et al., 2015) our developed hybrid peptide (CaD23) demonstrated at least comparable or better *in vitro* efficacy than these peptides. In addition, the encouraging *in vivo* efficacy and safety results further reinforced the translational potential of CaD23. Furthermore, it is interesting to note that the haemolytic activity of CaD23 was very low (7.1% at 200 μg/ml), suggesting that it can potentially be developed for treating systemic infection. It also reduces the concern of systemic toxicity when it is applied to the ocular surface because systemic absorption of ocular topical treatment can occur, particularly when the drugs are hydrophobic (Farkouh et al., 2016). The considerable disparity between the haemolytic activity and cytotoxicity against corneal epithelial cells could be attributed to the difference in the membrane structure (particularly the cytoskeleton) and the metabolic structures of the non-nucleated cells (e.g. red blood cells) and nucleated cells (e.g. corneal epithelial cells) (Smith, 1987). Further studies examining the efficacy and stability of CaD23 in serum as well as its interaction with blood proteins will be conducted to determine its therapeutic potential for treating systemic infection. In addition, as CaD23 demonstrated good safety at 0.05% but considerable toxicity at 0.1%, examining the efficacy and safety of CaD23 in intermediate concentrations such as 0.06% and 0.08% may provide further insight into the optimal concentration for clinical translation.

In conclusion, this work demonstrated that rational hybridisation of LL-37 and HBD-2 serves as a novel strategy in translating the therapeutic potential of human-derived HDPs. Future work examining the efficacy and safety of combined CaD23-antibiotic therapy would be beneficial. In addition, further optimisation of CaD23 using the strategies highlighted in Chapter 1 can potentially generate more efficacious and safer molecules than CaD23.

CHAPTER 8

Evaluation of Host Defense Peptide (CaD23)-Antibiotic Synergism and Mechanism of Action: Insights from Experimental and Molecular Dynamics Simulations Studies

8.1. Introduction

Antimicrobial resistance (AMR) is currently emerging as one of the major global health threats (Prestinaci et al., 2015, Ventola, 2015). By 2050, it is estimated to cause 10 million deaths and cost the global economy up to 100 trillion USD if the issue remains untackled (O'Neill, 2016). In view of the colossal impact on global health, various initiatives and strategies have been proposed and implemented to tackle AMR. These include establishment of antimicrobial stewardship to monitor the use of antimicrobial agents and the rise of AMR, development of new drugs and vaccines, drug repurposing, and incentivising pharmaceutical companies for investing in antimicrobial drug development (Ventola, 2015).

In the previous chapter, CaD23 was shown to exhibit a more rapid *in vitro* antimicrobial action than conventional antibiotics such as amikacin (Ting et al., 2021e). However, the mechanism of action has not been fully elucidated. In addition, while CaD23 was able to demonstrate moderate in vivo efficacy at a concentration of 0.05% (500 μg/ml), the use of a higher concentration of CaD23 (for higher antimicrobial effect) was prohibited by the toxicity observed in the wound healing

study. Therefore, to overcome this limitation, this study aimed to examine the potential synergism / interaction between CaD23 and conventional antibiotics that are commonly used for infectious keratitis (IK). In addition, this study also aimed to examine the mechanism of action of CaD23 using a combination of biophysical and molecular dynamics (MD) simulations studies (Ting et al., 2021k).

8.2. Materials and methods

8.2.1. Chemicals and antibiotics

All the peptides were commercially produced by Mimotopes (Mimotopes Pty. Ltd., Mulgrave Victoria, Australia) via traditional solid phase Fmoc synthesis method. All the synthetic peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) to >95% purity and characterised by mass spectrometry. The peptides were subsequently sent to our lab in lyophilised form and stored at -80 °C until further usage. In view of the hydrophobicity, CaD23 (sequence: KRIVQRIKDWLRKLCKKW; see Table 7.1) was first fully dissolved in 50 μl of dimethyl sulfoxide (DMSO) followed by dilution in sterile, de-ionised water to achieve a final concentration of 1 mg/ml peptide in 0.5% DMSO. Further dilution was performed for specific assays as required. All the assays described in this study were conducted in biological duplicate and in at least two independent experiments, with appropriate positive controls (PCs) and negative controls (NCs). Antibiotics, including levofloxacin and amikacin, were purchased from Sigma-Aldrich (Merck Life Science UK Ltd., Dorset, UK).

8.2.2. Types of microorganisms used

A range of Gram-positive and Gram-negative bacteria were used in this study. These included laboratory-strain methicillin-sensitive *Staphylococcus aureus* (SH1000 and

ATCC SA29213), methicillin-resistant *S. aureus* (ATCC MRSA43300), *Pseudomonas aeruginosa* ATCC PA19660 (cytotoxic strain), and *P. aeruginosa* ATCC PA27853 (invasive strain). Both cytotoxic and invasive *P. aeruginosa* strains were used in the experiments as previous studies had demonstrated the difference in virulence (Borkar et al., 2013, Lee et al., 2003).

8.2.3. Determination of *in vitro* antimicrobial efficacy

In vitro antimicrobial efficacy of CaD23 and the antibiotics was determined using the established minimum inhibitory concentration (MIC) assay with broth microdilution method approved by the Clinical and Laboratory Standards Institute (CLSI) as described in Chapter 7 (Clinical & Laboratory Standards Institute (CLSI), 2019).

8.2.4. Determination of the peptide-antibiotic interaction

The peptide-antibiotic interaction was determined using two methods, namely the checkerboard assay and the time-kill kinetics assay.

8.2.4.1. Checkerboard assay

The peptide-antibiotic synergism was examined using the established checkerboard assay described in the previous study (Mohammed et al., 2019). A 96-well polypropylene plate (Plate A) was used to prepare 8 replicate horizontal rows of CaD23 in twofold serial dilutions [from 400 μg/ml (1st column) to 6.25 μg/ml (7th column), and caMHB in the last (8th) column; final volume of 25 μl per well]. Another 96-well polystyrene plate (Plate B) was used to prepare 8 replicate vertical columns of an antibiotic, either amikacin (an aminoglycoside) or levofloxacin (a fluoroquinolone), in twofold serial dilutions [from 20 μg/ml (1st row) to 0.313 μg/ml (7th row), and 0 μg/ml in the last (8th) row; final volume of 30 μl per well]. Subsequently,

25 μl of antibiotic from each well of Plate B was transferred to the corresponding wells of Plate A (1:1 ratio of peptide and antibiotic). The bacterial suspension was prepared as above and 50 μl of $1x10^6$ CFU/ml bacteria was added into each well (1:1 ratio of treatment and bacteria; final concentration of 5×10^5 CFU/ml bacteria per well). The final concentration of CaD23 in each row was 100 μg/ml (1st column) to 1.56 μg/ml (7th column) and the final concentration of antibiotic in each column was 5 μg/ml (1st row) to 0.078 μg/ml (7th row). Growth control and sterility control were included in each experiment. The MIC was calculated as above after 18-21 hours of incubation with treatment at 37ºC.

The fractional inhibitory concentration index (FICI) is calculated using the formula: $(MIC_{CaD23(combined)} / MIC_{CaD23(alone)}) + (MIC_{antibiotic(combined)} / MIC_{antibiotic(alone)})$ and was interpreted as synergistic (FICI <0.5), additive (FICI between 0.5-1.0), indifferent (FICI between >1.0 and ≤ 4), or antagonistic (FICI >4).

8.2.4.2. Time-kill kinetics assay

Time-kill kinetics assay was performed to determine the time and concentration-dependent antimicrobial activity of CaD23 and amikacin against SH1000. The bacterial suspension (with a concentration of 1×10^6 CFU/ml) was prepared using the similar method as described in the MIC assay. 50 μl of bacteria was then incubated with 50 μl of respective treatment, consisting of either CaD23 alone, amikacin alone, or combined CaD23-amikacin. Bacterial suspension incubated with sterile de-ionised water (dH_2O) in 1:1 ratio was used as the growth control. At 0 min, 15 min, 30 min, 1 hour, 2 hour, 4 hours, and 24 hours, 10 μl of the treatment / bacteria mixture was removed from each well and was serially diluted (1:10 dilution) in sterile phosphate buffered saline (PBS). The diluted suspension (20 μl) was subsequently removed and

plated on Muller-Hinton agar (MHA) in duplicate for bacterial counting after incubation for 18-21 hours at 37ºC.

8.2.5. Evaluation of the mechanism of action

8.2.5.1. SYTOX green uptake assay

SYTOX green is a membrane-impermeable dye that activates and fluoresces upon binding to the DNA. The assay was performed using a previously established method, with a slight modification (Mayandi et al., 2020). Briefly, the bacteria were cultured overnight in MHB (20 μl) for 16-18 hours. Subsequently, the bacterial suspension was vortexed, washed twice and suspended in sterile HEPES buffer solution (5 mM HEPES, 5 mM glucose, 7.4 pH) to obtain an OD_{600} of 0.3. An aliquot of 5 mM SYTOX green stock solution in DMSO was added to the bacterial suspension to obtain a final dye concentration of 2 μM. The mixture was incubated for 15 minutes at room temperature while being protected from light. The dye-loaded cell suspension (600 μl) was then added into a stirring quartz cuvette and inserted into a QuantaMaster spectrofluorometer for fluorescence time-based scan at 504 nm excitation and 523 nm emission. Once a constant fluorescence level was achieved, a concentrated peptide solution in water (1 μl) was added into the cuvette to obtain a desired final concentration of CaD23 (2x MIC) in the cell suspension. The change in fluorescence intensity was monitored until a stable range was observed. Maximum fluorescence was documented via the addition of Triton-X (final concentration of Triton-X 0.1% (v/v) in 600 μl cell suspension) into the cuvette. The fluorescence intensity (I) of the peptide-treated suspension was calculated and plotted as: $(I_{peptide} / I_{Triton\text{-}X(max)}) \times 100\%$

8.2.5.2. Molecular dynamics simulations

Established molecular dynamics (MD) simulations-based models were used to examine the interactions between the synthetic peptides and models of the bacterial

and mammalian membranes, using the GROMACS 5.1 package.[16] In view of the specialised nature, this part of the work was conducted with the help of Dr. Jianguo Li, who was the post-doctoral researcher in the A*Star Institute in Singapore specialising in computational simulation.

The ability of peptide to permeate or interact with the bacterial membrane and mammalian membrane served as a proxy for its antimicrobial efficacy and toxicity, respectively. The bacterial membrane was modelled using a mixture of phosphoethanolamine and phosphatidylglycerol lipids (3:1 ratio) whilst the mammalian membrane was modelled using phosphotidylcholine. Each membrane patch consists of 128 lipid molecules. The peptide was modelled using the AMBER14sb force field, and the lipid molecules were modelled using the AMBER lipid17 force field. Initially, the peptide, modelled in a helical conformation, was placed 4 nm above the membrane center, followed by solvation with water molecules using the TIP3 model of each system (Jorgensen et al., 1983). Counter ions were added to neutralise each system. Each system was first subjected to 500 steps of energy minimisation, followed by 20 ps of MD simulation in the canonical NVT ensemble (N = constant number; V = volume; T = temperature). Each system was first simulated for 400 ns to allow the peptide to adsorb on the membrane surface. Due to the complex free energy landscape of the peptide-membrane system, the time scale required to reach the equilibrium state was considerably lengthy. To overcome this difficulty, 400 ns simulations of simulated annealing, as outlined by Farrotti et al. (2015), were performed. In each simulated annealing cycle, the temperature of the system was increased from 300 K to 375 K in 50 ps steps, followed by a 1 ns simulation at 300 K. This was followed by 400 ns of normal MD simulation at 300 K. The LINCS algorithm (Hess et al., 1998) was applied to restrain the bond between hydrogen atoms and heavy atoms, enabling a time step of 2 fs. Both Lennard-Jones and short-range electrostatic interactions were set to extend to 0.9 nm, while the long

range electrostatic interactions were calculated using particle mesh Ewald method (Essmann et al., 1995). The temperature and pressure were controlled by Nose–Hoover (Nose and Klein, 1983) and semi-isotropic Parrinello–Rahman algorithms (Martonák et al., 2003), respectively.

8.3. Results

8.3.1. Peptide-antibiotic interaction

8.3.1.1. Checkerboard assay

The MICs of CaD23, amikacin and levofloxacin against Gram-positive and Gram-negative bacteria are presented in **Table 8.1**. A number of peptide-antibiotic combinations were examined for their interactive antimicrobial effect against both Gram-positive and Gram-negative bacteria (**Table 8.2**). It was found that both CaD23-amikacin and CaD23-levofloxacin combinations achieved strong additive effects against SH1000 (FICI = 0.563) and MRSA43300 (FICI = 0.563). On the other hand, the effect of CaD23-amikacin was indifferent against PA19660 (FICI = 1.08) and borderline additive against PA27853 (FICI = 1.0), whereas the effect of CaD23-levofloxacin against PA19660 and PA27853 was indifferent (both FICI = 2.0).

Table 8.1. Minimum inhibitory concentration (MIC) of CaD23 and antibiotics.

Treatment	SH1000	MRSA43300	PA19660	PA27853
CaD23	12.5	25	25	25
Amikacin	1.25	2.5	0.63	1.25
Levofloxacin	0.31	0.31	0.31	0.63

All assays were conducted as two independent experiments in biological duplicate. Various organisms, including methicillin-sensitive *S. aureus* (SH1000), methicillin-resistant *S. aureus* (ATCC MRSA43300), *P. aeruginosa* ATCC PA19660 (cytotoxic strain) and ATCC PA27853 (invasive strain), were used. The MIC value is expressed in μg/ml.

Table 8.2. Interactive antimicrobial effect of CaD23 and antibiotics using checkerboard assay.

Treatment	Bacteria	FICI*	Interpretation
CaD23 + Amikacin	SH1000	0.563	Additive
	MRSA43300	0.563	Additive
	PA19660	1.08	Indifferent
	PA27853	1.0	Borderline additive
CaD23 + Levofloxacin	SH1000	0.563	Additive
	MRSA43300	0.563	Additive
	PA19660	2.0	Indifferent
	PA27853	2.0	Indifferent

MIC = Minimum inhibitory concentration; FICI = Fractional inhibitory concentration index

*FICI is calculated as: ($MIC_{CaD23(combined)}/MIC_{CaD23(alone)}$) + ($MIC_{ami(combined)}/MIC_{ami(alone)}$)

FIC $\leq$ 0.5 = synergistic; FIC between 0.5-1.0 = additive; FIC >1 to 4 = indifferent; FIC>4 = antagonistic.

Experiments were conducted against methicillin-sensitive *S. aureus* (SH1000), methicillin-resistant *S. aureus* (ATCC MRSA43300), and *P. aeruginosa* ATCC PA19660 (cytotoxic strain) and ATCC PA27853 (invasive strain).

The results are based on two independent experiments performed in biological duplicate.

8.3.1.2. Time-kill kinetics assay

Based on the results of the checkerboard assay, the concentration- and time-dependent antimicrobial effect of combined CaD23-amikacin against SH1000 was further explored. The MIC of CaD23 and amikacin against SH1000 was 12.5 μg/ml and 1.25 μg/ml, respectively. When CaD23 was used alone at the concentration of 25 μg/ml (2x MIC), it was able to achieve 99.9% and 100% killing of SH1000 by 15 mins and 30 mins post-treatment, respectively (**Figure 8.1**). This was significantly faster than amikacin at 10 μg/ml (4x MIC) or 25 μg/ml (10x MIC), which both achieved 99.9% and 100% killing of SH1000 by 2 hours and 4 hours post-treatment (i.e. 8 times slower). The addition of CaD23 (3.1 μg/ml; 0.25x MIC) expedited the antimicrobial action of amikacin (10 μg/ml) against SH1000 by 4 times (for 99.9% killing) and 2 times (for 100% killing). In addition, combined CaD23 (3.1 μg/ml; 0.25x MIC) and amikacin (2.5 μg/ml; 2x MIC) was able to achieve 99.9% and 100% killing by 1 hour and 2 hours, respectively. This was 2 times faster than amikacin when used alone at 10 μg/ml or 25 μg/ml, suggesting that combination treatment enables a more effective killing and lowers the treatment concentration required for effective killing.

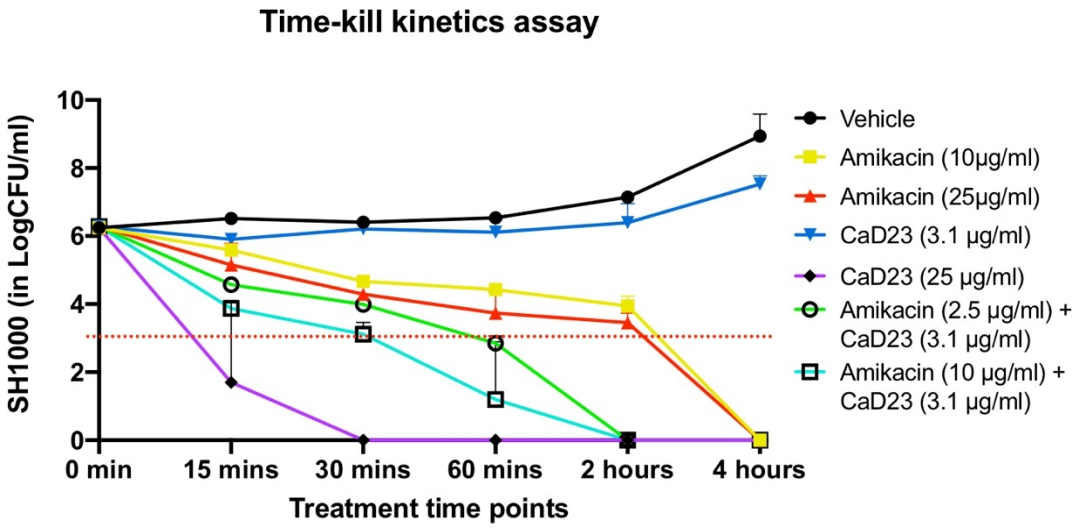


Figure 8.1. Time-kill assay for CaD23, amikacin and combined treatment.

Time- and concentration-dependent anti-bacterial effect of CaD23 (0.25x MIC and 2x MIC), amikacin (8x and 20x MIC) and combined CaD23-amikacin against *S. aureus* (SH1000) over 24 hours. SH1000 incubated with phosphate buffer saline (PBS) serves as the untreated control. "0 min" represents the starting inoculum, which is around 6 $\log_{10}$ CFU/ml. The red dotted horizontal line at 3 $\log_{10}$ CFU/ml signifies the threshold of significant bacterial killing (defined as 99.9% or 3 $\log_{10}$ CFU/ml reduction of the bacterial viability compared to the starting inoculum). Data is presented as mean ± standard deviation (depicted in error bars) of two independent experiments performed in biological duplicate.

8.3.2. Mechanism of action of CaD23

8.3.2.1. SYTOX green uptake assay

SYTOX green uptake assay was performed to study the underlying mechanism of action of CaD23 against *S. aureus* ATCC SA29213 (MIC = 25 μg/ml). It was shown that CaD23 at 50 μg/ml (2x MIC) exhibited rapid membrane permeabilisation of SA29213, with 60% SYTOX green uptake observed within seconds of treatment and reaching 80% membrane permeabilisation at around 8 mins post-treatment (**Figure 8.2**).

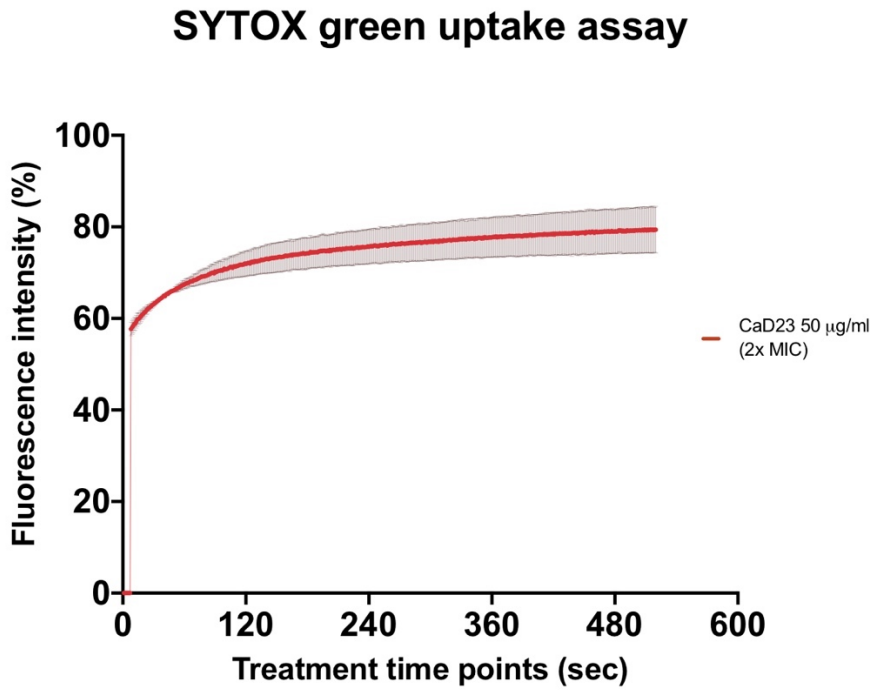


Figure 8.2. Membrane permeabilising action of CaD23 determined by SYTOX green uptake assay.

The graph demonstrating rapid membrane permeabilising action of CaD23 (50 μg/ml; 2x MIC) against SA29123, with a 60% increase in fluorescence intensity (due to SYTOX green uptake) within seconds of treatment and plateaued at ~80% fluorescence intensity at 8 minutes. The fluorescence intensity is presented as mean ± standard deviation (depicted in error bars) of two independent experiments. The maximum fluorescence intensity (100%) was derived from the positive control, Triton-X 0.1% (v/v). Fluorescence intensity (I) of the peptide-treated suspension was calculated and plotted as: $(I_{peptide} / I_{Triton\text{-}X(max)}) \times 100\%$.

8.3.2.2. Molecular dynamics (MD) simulations

To understand the mode of interactions of the CaD23 peptide with the membranes, MD simulations of CaD23 with model bacterial and mammalian membranes were carried out. The distance between the centre of mass of CaD23 and the bilayer centre of mammalian and bacterial membranes is shown in **Figure 8.3A-B**. In the first 400 ns, the distance between CaD23 and both membranes decreased, suggesting a rapid adsorption of CaD23 on both membranes. CaD23 was closer to the bacterial membrane (z-distance = 2 nm) than the mammalian membrane (z-distance = 3.5 nm with considerable fluctuation), suggesting a stronger peptide-bacterial membrane interaction. The different locations of CaD23 with respect to the bilayer center can also be seen from the density distribution of CaD23 with respect to the phosphate atoms during the final 400 ns of the MD simulations (**Figure 8.3C-D**). On the mammalian membrane, the peak of CaD23 was low and the distribution of CaD23 was wide and far away from the phosphate groups, suggesting a low affinity of CaD23 to the mammalian membrane. In contrast, the peak of CaD23 was close to the phosphate groups upon strong adsorption on the bacterial membrane.

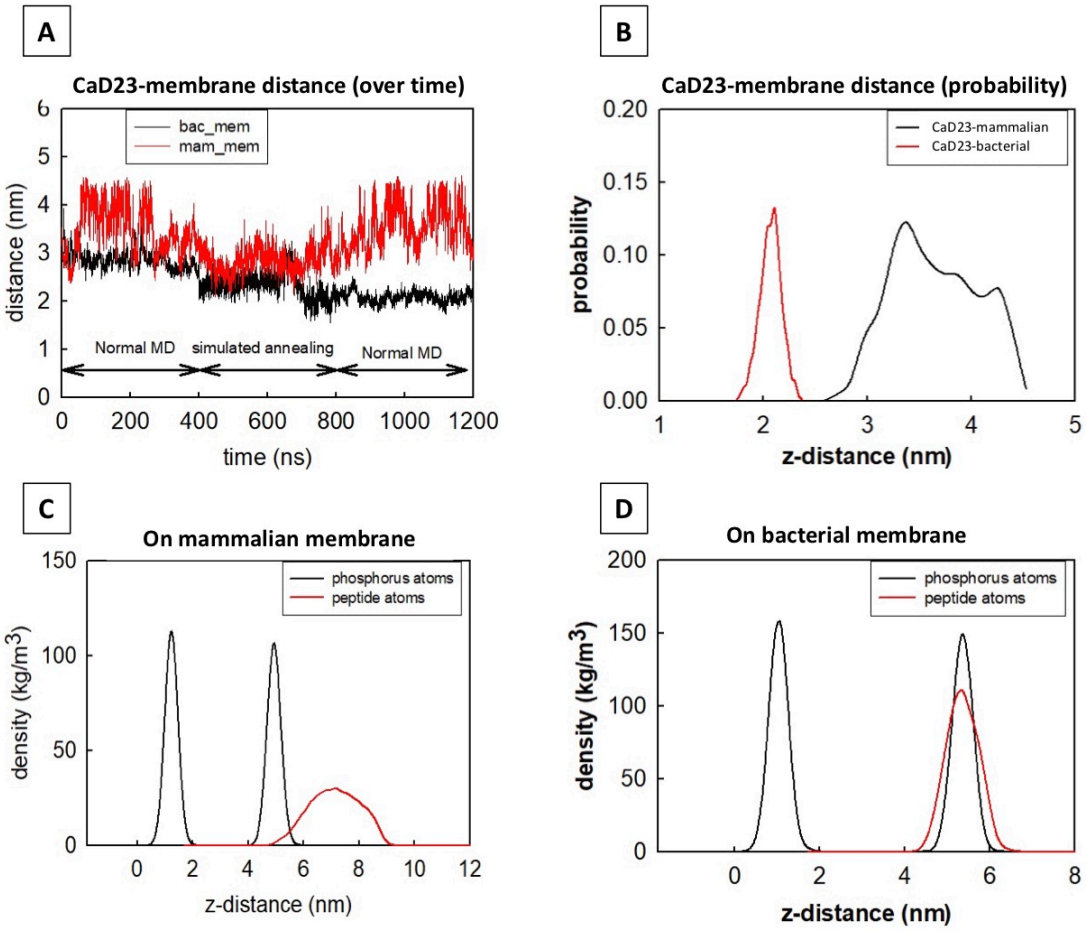


Figure 8.3. MD simulation of CaD23 on model mammalian and bacterial membranes. Each simulation was run for 400 ns at 300 K, followed by another 400 ns using simulated annealing (SA) to accelerate phase space sampling, finally followed by a further 400 ns simulation to obtain equilibration. **(A)** The graph showing the distance between CaD23 and mammalian or bacterial membrane over 1200 ns. CaD23 is shown to be closer to the bacterial membrane than to the mammalian membrane, suggesting a stronger interaction between CaD23 and the bacterial membrane. **(B)** The probability distribution of the peptide-membrane distance in the last 400 ns, demonstrating a closer distance of CaD23 to the bacterial membrane than to the mammalian membrane. **(C-D)** Density distributions of the CaD23 with respect to the phosphate groups of the bilayer membranes. The analysis is based on the last 400 ns simulation.

Representative snapshots of the MD simulations of CaD23 with mammalian and bacterial membranes are shown in **Figure 8.4**. Upon adsorption on the membrane, CaD23 started to interact with the head groups of the membrane, which involved the rearrangement of the head groups and the penetration of hydrophobic residues of CaD23 into the membrane (**Figure 8.4**). Due to complex mode of interactions, this process was characterised by a frustrated free energy landscape. To accelerate sampling, simulated annealing (SA) was applied. The peptide-membrane distance was found to decrease further, particularly for the distance between CaD23 and the bacterial membrane, because the strong perturbation of the bacterial head groups facilitated the penetration of the hydrophobic residues of CaD23 into the lipid tail region of the bacterial membrane, which did not occur on the mammalian membrane due to weak interactions. To obtain an equilibrium state, classical MD simulations without SA were carried out for a further 400 ns. The distance between the peptide and the bacterial membrane decreased further and remains stable. In contrast, the distance between CaD23 and the mammalian membrane increased and fluctuated with many adsorption-desorption events on the mammalian membrane, suggesting a weaker interaction.

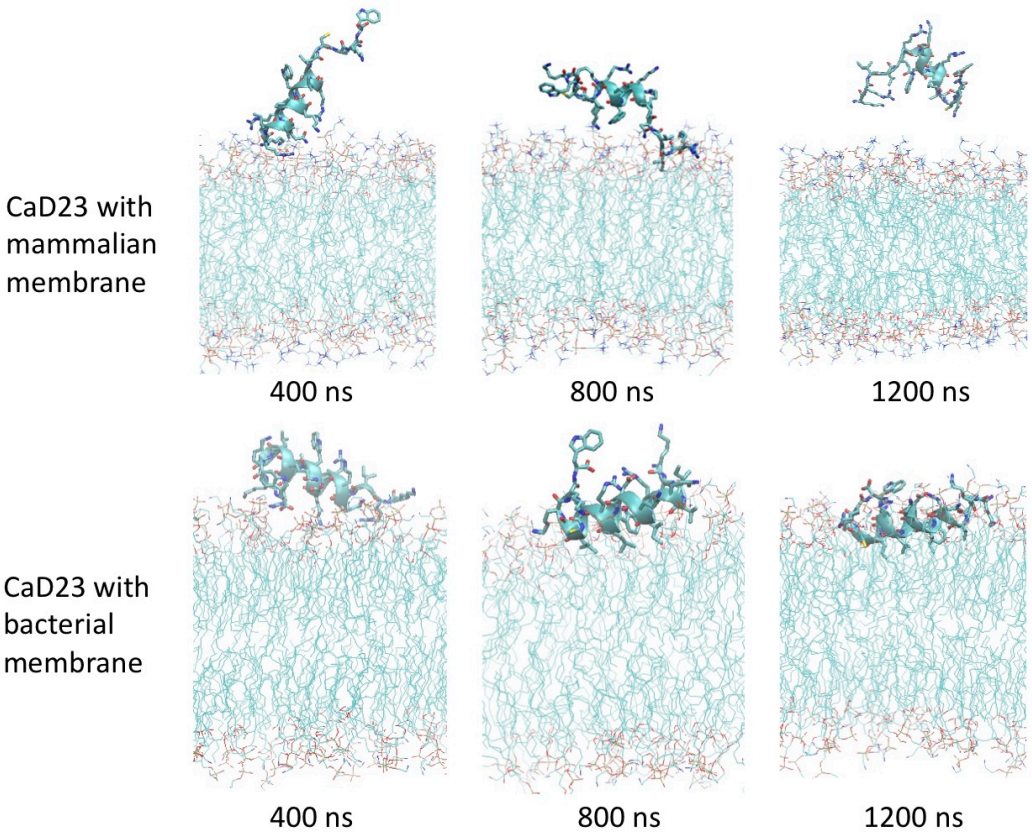


Figure 8.4. Representative snapshots of CaD23 with mammalian and bacterial mimetic membranes.

The conformation of each snapshot corresponds to the 1st cluster of CaD23 during the last 200 ns simulations.

The helical wheel revealed that when the peptide was in helical conformation, it formed a perfect facial amphiphilic conformation, with positively charged residues facing one side and the hydrophobic residues facing the other side (**Figure 8.5**). Although the peptide largely maintained the helical conformation on both membranes, the peptide was more helical on the bacterial membrane than on the mammalian membrane. The snapshots from the last 400 ns in **Figure 8.4** clearly demonstrate that the peptide adopts a helical conformation on the bacterial membrane, with the hydrophobic residues inserted into the lipid tail region while the basic residues interact with the head groups, resulting in perturbation of the membrane-water interface. On the mammalian membrane, CaD23 was only partially helical and fluctuated due to the lack of strong electrostatic interactions, resulting in less perturbation of the mammalian membrane. This explains why CaD23 has a higher antimicrobial activity than toxicity (therapeutic index of >8 against *S. aureus*). Moreover, CaD23 formed more hydrogen bonds with the bacterial membrane compared to the mammalian membrane (**Figure 8.6**), which further contributed to the high affinity towards bacterial membrane.

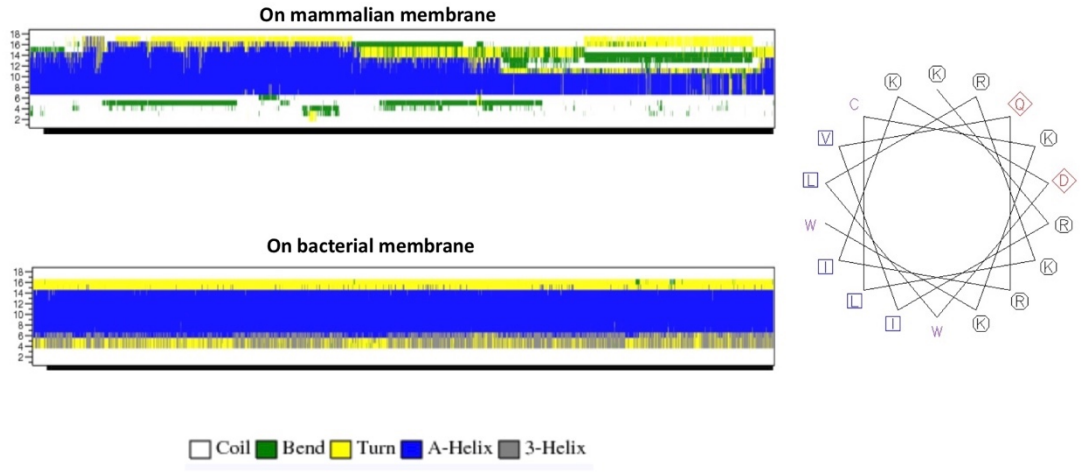


Figure 8.5. Prediction of secondary structure using MD simulations.
(A) Secondary structure evolution of CaD23 during the molecular dynamics (MD) simulations. CaD23 adopts a partially alpha-helical structure on the mammalian membrane compared to a highly alpha-helical structure on the bacterial membrane. **(B)** The helical wheel plot of CaD23. Blue and purple letters represent hydrophobic residues, red letters represent negatively charged acidic residues, and black letters represent positive charged basic residues.

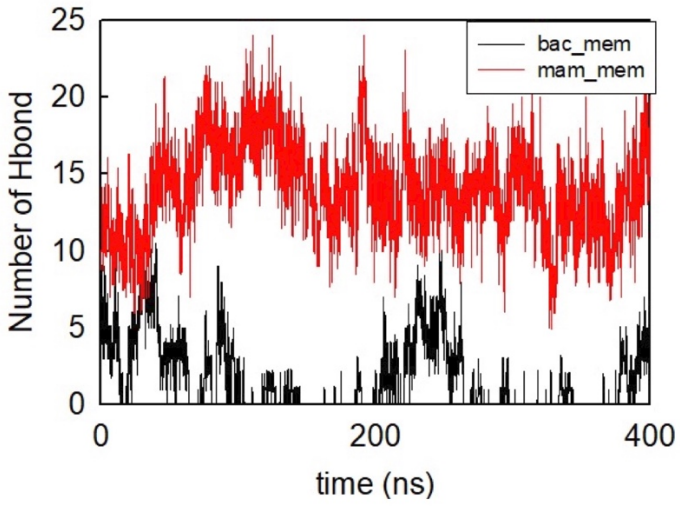


Figure 8.6. Number of hydrogen bond formed between CaD23 and the membranes during the last 400 ns.

8.4. Discussion

The serendipitous discovery of HDPs dates back to as early as 1920s where lysozyme was first discovered by Sir Alexander Fleming. However, the research interest in HDP-based antimicrobial therapy was only reinvigorated in 1980s during the wake of multidrug-resistant microbial pathogens and improvement in the isolation and characterisation techniques of HDPs (Wang, 2014). HDPs have been shown to exhibit broad-spectrum and rapid antimicrobial action, with low risk of developing AMR, but a number of barriers, including toxicity to host cells / tissues, have so far impeded the translation of HDP-based treatment to clinical use. In this study, we demonstrated that CaD23 could enhance the antimicrobial efficacy of commonly used antibiotics, including amikacin and levofloxacin, in a strong additive manner, against methicillin-sensitive and methicillin-resistant *S. aureus* when they were used in combination. This suggests that a lower treatment concentration of CaD23 and antibiotic can be used, serving as a useful strategy to reduce the concentration-dependent drug toxicity that is often observed in clinical practice (Dua et al., 2012, Forster et al., 2005).

Furthermore, the addition of CaD23 at sub-MIC level was able to expedite the antimicrobial action of amikacin by 2-4 times when used in combination. Theoretically, such beneficial effect can reduce the risk of developing AMR as the bacteria have less time to adapt and develop effective mechanisms against the antibiotics. Studies have shown that membrane-active peptides with rapid antimicrobial action have a low risk of developing AMR whereas conventional antibiotics are prone to developing AMR, especially when they are chronically used at a sub-MIC level (Mayandi et al., 2020, Hancock and Sahl, 2006, Llor and Bjerrum, 2014). This is due to the fact that modification of the entire membrane of the microorganisms in response to membrane-active peptides incurs a high fitness cost when compared to alteration of

a particular binding site targeted by conventional antibiotics (e.g. alteration in the penicillin-binding protein reduces the efficacy of beta-lactam antibiotics) (Kapoor et al., 2017, Ting et al., 2020a).

The advantageous strong additive effects of CaD23-amikacin and CaD23-levofloxacin against Gram-positive bacteria are likely attributed the different underlying mechanism of action of these drugs. Amikacin is a commonly used aminoglycoside in clinical practice (including ophthalmology) that exhibits its antimicrobial activity via inhibition of the 30S ribosomal subunit whereas levofloxacin, a frequently used fluoroquinolone, kills bacteria by inhibiting the bacterial DNA gyrase (Krause et al., 2016, Ting et al., 2021h). It is likely that CaD23 interacts and permeabilises the cytoplasmic membrane of Gram-positive bacteria and facilitates the penetration of aminoglycoside and levofloxacin into the bacterial cells, enabling a more effective binding to the intracellular targets. However, none of the combinations exhibited an antagonistic effect, suggesting that both treatment work on separate targets.

Interestingly, we did not observe the same antimicrobial additive effect when CaD23 was used in combination with either amikacin or levofloxacin against Gram-negative bacteria. One of the main differences between Gram-positive and Gram-negative bacteria lies in the different compositions of the bacterial cell envelope (Silhavy et al., 2010). While both types of bacteria have a cytoplasmic / inner membrane, Gram-positive bacteria possess a thick peptidoglycan outer layer whereas Gram-negative bacteria possess an additional outer membrane, which is primarily composed of negatively charged lipopolysaccharides (in the outer leaflet of the outer membrane) (Kapoor et al., 2017). It is likely that CaD23 primarily acts on the inner cell membrane (of both types of bacteria), with a weaker interaction with lipopolysaccharides, thereby

explaining the additive effects of combined CaD23-antibiotic that were observed in Gram-positive bacteria but not in Gram-negative bacteria.

On the other hand, our group had recently demonstrated that FK16 (a truncated version of LL-37) was able to enhance the antimicrobial activity of vancomycin against *P. aeruginosa*. Vancomycin is a glycopeptide antibiotic that has poor permeability against the outer membrane of Gram-negative bacteria (Antonoplis et al., 2019). It was hypothesised that FK16, a membrane-active peptide, permeabilises the outer membrane of the Gram-negative bacteria and improves the delivery of vancomycin to access periplasmic cell wall precursors and intracellular target. Antonoplis et al. (2019) had similarly demonstrated the synergistic effect in a vancomycin-arginine peptide conjugate in treating carbapenem-resistant *Escherichia coli*, likely through a similar mechanism of action described above. Kampshoff et al. (2019) observed a synergistic effect between ciprofloxacin and melimine (a highly cationic, hybridised peptide derived from melittin and protamine) against ciprofloxacin-resistant *P. aeruginosa*, but not against *S. aureus* or non-drug resistant *P. aeruginosa*. In addition, a synergistic effect was not observed in either melimine-cefepime (a fourth-generation cephalosporin), Mel4 (truncated melimine)-ciprofloxacin, or Mel4-cefepim, highlighting the heterogeneous interactions among different types of peptides and antibiotics.

Both SYTOX green uptake assay and MD simulation studies demonstrated that CaD23 achieved its antimicrobial activity via a membrane-permeabilising action. In the recent decades, MD simulations have been increasingly utilised in the process of drug discovery and development in many fields, including the field of HDPs (Durrant and McCammon, 2011, De Vivo et al., 2016, Li et al., 2017, Ting et al., 2020a, Li et al., 2012). They have been shown to predict the secondary structures of proteins / peptides, decipher the underlying mechanism of action, and identifying key residues

responsible for the protein-protein or protein-membrane interaction at an atomistic level (Li et al., 2017, Li et al., 2018, Tsai et al., 2009). As the chemical space of synthetic and natural HDPs is vast, MD simulation serves as a powerful tool to expedite the process of designing and optimising the peptide sequences as it reduces the need for repetitive microbiological assays and laborious screening of a large amount of peptide that is usually required in traditional mutation-based empirical methods.

A number of key factors, including alpha-helicity, amphiphilicity, cationicity and hydrophobicity, have been described to influence the antimicrobial efficacy of HDPs (Ting et al., 2020a, Hancock and Sahl, 2006, Mookherjee et al., 2020). In our study, MD simulations have revealed a number of important findings pertaining to the CaD23 molecule. Firstly, we observed a rapid adsorption of CaD23 on the negatively charged bacterial membrane during the early stage of the simulation (particularly at the N-terminus where the Lys1 is located), highlighting the importance of cationicity in the CaD23 molecule. In contrast, the zwitterionic nature of the mammalian membrane exhibited a weaker interaction with CaD23. Secondly, we showed that CaD23 adopted a more alpha-helical conformation on the bacterial membrane than the mammalian membrane, suggesting that alpha-helicity plays an important contributory role to the antimicrobial efficacy of CaD23. In the helical conformation, the peptide displays high facial amphiphilicity, which resulted in a more favourable interaction with the bacterial membrane, with a deeper penetration of CaD23 into the bacterial membrane. This is in accordance with many studies in the literature that had highlighted the important correlation between alpha-helicity and antimicrobial efficacy observed in various natural and synthetic HDPs (Haney et al., 2019, Mookherjee et al., 2020). The Trp18 residue at the C-terminal was shown to have a strong interaction with the bacterial membrane but not the Trp10 residue. This suggests that the Trp10 residue may potentially be substituted with a less hydrophobic residue such

as Leu or Ile to reduce the hydrophobicity and toxicity, and to improve its water solubility.

Despite the many advantages of MD simulations described above, it is noteworthy to mention that the model bacterial membrane utilised in the current MD simulation is only representative of the inner membrane of the Gram-positive and Gram-negative bacteria. Atomistic models have been developed for bacterial outer membrane and several studies have been carried out to understand the structural dynamics of the outer membrane (Pontes et al., 2012, Li et al., 2020a, b). However, MD simulations with outer membrane is out of the scope of this study as CaD23 was mainly efficacious against Gram-positive bacteria.

In summary, this work demonstrated that CaD23 is a membrane-active peptide that has the ability to enhance the antimicrobial action of commonly used antibiotics such as amikacin and levofloxacin, potentially offering a new therapeutic strategy for Gram-positive bacterial infection. Further in vivo studies to validate these results would be invaluable. In addition, MD simulation serves as a useful computational tool in deciphering the underlying mechanism of action and guiding the design process of HDPs.

CHAPTER 9

CONCLUSIONS

9.1. Discussion

Infectious keratitis (IK) represents a significant ocular morbidity in both developed and developing countries. In this work, a multi-pronged approach was employed to advance the knowledge and treatment of IK, including: (1) an up-to-date review of IK and host defense peptides (HDPs); (2) a comprehensive analysis of the epidemiological and clinical aspects of IK in Nottingham, UK; (3) systematic reviews of current and emerging adjuvant surgical procedures for IK; and (4) developing a new therapeutic approach for managing IK using HDP-based treatment. The significance of this body of work is discussed below.

9.2. Significance of the work

9.2.1. The Nottingham Infectious Keratitis Study

In Chapters 2-4, an extensive and comprehensive analysis of the epidemiology, causative microorganisms, risk factors, clinical characteristics, management, outcome and prognostic factors of IK in Nottingham, UK, over the past decade was performed. To date, there were only two studies that had examined the incidence of IK in the UK, which was estimated at 3.6 – 52.1 per 100,000 population/year during the period of 1995 – 2010 (Seal et al., 1999, Ibrahim et al., 2012). However, these studies were performed more than a decade ago. This recent Nottingham Infectious Keratitis Study serves as the most up-to-date examination of the incidence of IK in the UK over the past decade. The incidence was estimated at 34.7 per 100,000 population in Nottingham, UK, over the past decade, which was similar to the findings

of the previous studies, suggesting that IK represents a persistent burden on human health in the UK. However, it is noteworthy to highlight that the incidence was estimated based on patients who had undergone corneal scraping for presumed IK. Therefore, the true incidence of all types of IK was likely to be underestimated as cases of milder IK or viral keratitis are usually managed without any corneal scraping. In addition, the culture yield was only 36%, which was a common issue highlighted in many other IK studies. This not only affects the evaluation of the underlying organisms but also affects the choice of antimicrobial treatment, particular in patients that do not respond to the initial broad-spectrum antimicrobial therapy. Increased effort needs to be invested in the diagnostic modality of IK in the near future to enable a more effective antimicrobial treatment in clinical practice. Reassuringly, the current broad-spectrum antimicrobial treatment provides a good antibiotic coverage to most bacteria, though ongoing surveillance of antimicrobial resistance is necessitated.

The Nottingham Infectious Keratitis Study also highlighted *Pseudomonas aeruginosa* and *Staphylococcus aureus* as the two most common organisms responsible for IK in Nottingham, which is similar to several other UK studies. Therefore, development of newer treatment targeting these common organisms is likely to reap the maximal benefit. This finding also supported the subsequent part of this work on developing a new HDP-based treatment focussing on *S. aureus* and *P. aeruginosa*.

In terms of clinical outcome, this study showed that the presenting vision and the initial severity of IK were the main prognostic factors for final visual outcome and corneal healing time. This highlights that prevention is more important than cure for IK. As ocular surface diseases and contact lens wear were shown to be the two most common risk factors, development of better preventative and monitoring strategies for these higher risk groups of patients are required.

9.2.2. Systematic reviews on adjuvant surgical treatment for infectious keratitis

In the Nottingham Infectious Keratitis Study, it was found that 16% of the patients required additional adjuvant surgical treatment. However, the effectiveness and safety of these adjuvant surgical treatment, including photoactivated chromophore for infectious keratitis-corneal collagen cross-linking (PACK-CXL) and amniotic membrane transplant (AMT), has not been fully assessed. To address this issue, two separate systematic reviews and meta-analyses were conducted to examine the role of adjuvant PACK-CXL and adjuvant AMT in IK, in addition to the standard antimicrobial treatment.

The meta-analyses demonstrated that these surgical procedures were able to expedite the corneal healing of IK in clinical setting, serving as useful adjuvant treatment for IK. However, the quality of evidence was low due to the risk of bias identified in the included randomised controlled trials (RCTs), highlighting the need for further high-quality RCTs. Another common problem is the lack of standardisation of the outcome reporting in IK clinical trials. The lack of high-quality evidence and the issues highlighted in these systematic reviews help form the basis for future RCTs in these areas.

9.2.3. Development of human-derived hybrid host defense peptides for treating infectious keratitis

Since the increased discovery of host defense peptides (HDPs) in nature during early 1980s, immense research effort has been invested in realising the therapeutic potentials of HDPs in clinic (Haney et al., 2019, Ting et al., 2020a, Wang, 2014). At the ocular surface, Dua's group was the first to discover and profile the spectrum of HDPs, including human cathelicidin (LL-37) and human beta-defensins (HBDs)-1 to

-3 (Haynes et al., 1998, 1999). Subsequently, the important roles of HDPs during IK were further observed and substantiated by several research groups (Haynes et al., 1998, Huang et al., 2007a, Huang et al., 2006, Huang et al., 2007b, Abedin et al., 2008, Dua et al., 2014, Mohammed et al., 2020, Mohammed et al., 2010, Mohammed et al., 2011). These novel observations have led to the conception of rational hybridisation of LL-37 and HBD-1 to -3 to enhance the therapeutic efficacy of these molecules.

In this work, a number of strategies and tools were employed to improve the therapeutic efficacy and safety of the designed hybrid peptides via a systematic approach, including modification of the cationicity, hydrophobicity, alanine scanning and C-terminal modification. In addition, a combination of experimental and molecular dynamics (MD) simulations studies were used to further examine the underlying mechanism of action and to identify the key residues responsible for the efficacy and toxicity. Finally, the developed hybrid HDP, named CaD23, was tested in established corneal wound healing and bacterial keratitis murine models, which demonstrated relatively good antimicrobial efficacy. Furthermore, CaD23 demonstrated a strong additive effect when used in combination with conventional antibiotics against Gram-positive bacteria, offering a potentially new therapeutic strategy in the future.

9.3. Future directions

While this work has generated a body of new and original findings in relation to IK, it has also created a number of new research questions that are worthy of further exploration and examination.

One of the main issues in current clinical practice is the low and variable culture yield, which can have a significant negative impact on the management of IK. So far, a

number of novel and emerging technologies, including matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (Singhal et al., 2015, Ting et al., 2020e), in vivo confocal microscopy (Chidambaram et al., 2018a), polymerase chain reaction (Somerville et al., 2020), next generation sequencing (Ung et al., 2020a), and artificial intelligence-assisted platforms (Ting et al., 2021c), have been developed and/or implemented in clinical practice. Future studies comparing the efficacy of these new technologies with the current conventional microbiological method would be invaluable.

So far, a number of HDP-based treatment have entered advanced (phase II/III) clinical trials, but none had reached the market due to regulatory hurdles (Fox, 2013, Boto et al., 2018). Nevertheless, many lessons have been learnt from past experience. One of the notable examples is MSI-78 or pexiganan, a magainin-derived HDP, which did not obtain the FDA approval after failing to demonstrate any superiority to the normal standard wound care with oral ofloxacin for infected diabetic foot ulcers in two phase 3 trials (Lipsky et al., 2008). Although the discouraging results have painted a gloomy outlook for HDP-based treatment at that time, a closer look at the development pathway of MSI-78 has shed light on the plausible reasons accounting for the failure. First, although the molecule demonstrated a broad-spectrum antimicrobial activity against 3109 clinical isolates (with an average MIC_{90} of 32 μg/ml or less) (Ge et al., 1999), the activity remained considerably weaker than the conventional antibiotics (Gottler and Ramamoorthy, 2009). Second, peptide-based treatment including MSI-78 are more susceptible to proteolytic degradation when compared to the conventional small molecule antibiotics. This suggests that HDP-based treatment needs to be administered in a higher concentration to achieve the intended *in vivo* efficacy, which could inevitably lead to increased host toxicity. In addition, MSI-78 exhibits several favourable properties over conventional antibiotics, including low risk of developing AMR and good activity against MDR isolates (Ge et

al., 1999), but the phase 3 clinical trials of MSI-78 were conducted for mild infective diabetic foot ulcers which did not fully capitalise on these strengths. This highlights the importance of setting the right research question during the development of HDP-based treatment.

Learning from the previous (unsuccessful) experience, a plethora of strategies have been proposed and attempted to overcome the inherent limitations of HDP-based treatment, with enhanced antimicrobial efficacy, proteolytic stability, and cell selectivity (for microbial cells). Although the design of ideal HDPs is not governed by a single overarching rule (Haney et al., 2019), it is apparent from the literature that peptide design guided by the fundamental principles and systematic SAR analysis is able to yield potential efficacious peptide candidates with desired properties (Hilpert et al., 2005). In fact, *de novo* designed synthetic peptides were successfully developed purely based on Arg and Trp with 50% hydrophobicity and demonstrated significantly antimicrobial and anti-biofilm efficacies against MDR staphylococci (Mohamed et al., 2016).

Based on the literature and the experience gained from this work, I have proposed a potential strategy in streamlining the drug discovery and development pathway of HDP-based treatment, starting from designing new HDP treatment to conducting well-designed pre-clinical studies (**Figure 9.1**). So far, more than 3000 naturally occurring and synthetic HDPs (with reported antimicrobial and/or non-antimicrobial functions) have been discovered (Wang et al., 2016, Zhao et al., 2013); therefore, it would be a good strategy to use an existing template with proven effect as a starting point for designing a new HDP-based treatment. Alternatively, employing artificial intelligence technology in predicting potentially efficacious molecules could be utilised. Once a starting template is identified (either a linear peptide or a cyclic peptide), systematic SAR analysis of the sequence via rational substitution of specific residues is required

to optimise the antimicrobial efficacy and cell selectivity toward microbial cells. If hybridisation strategy is used, functioning sequence of each single peptide should be first determined before being hybridised. This is then followed by further SAR analysis to determine the optimal sequence.

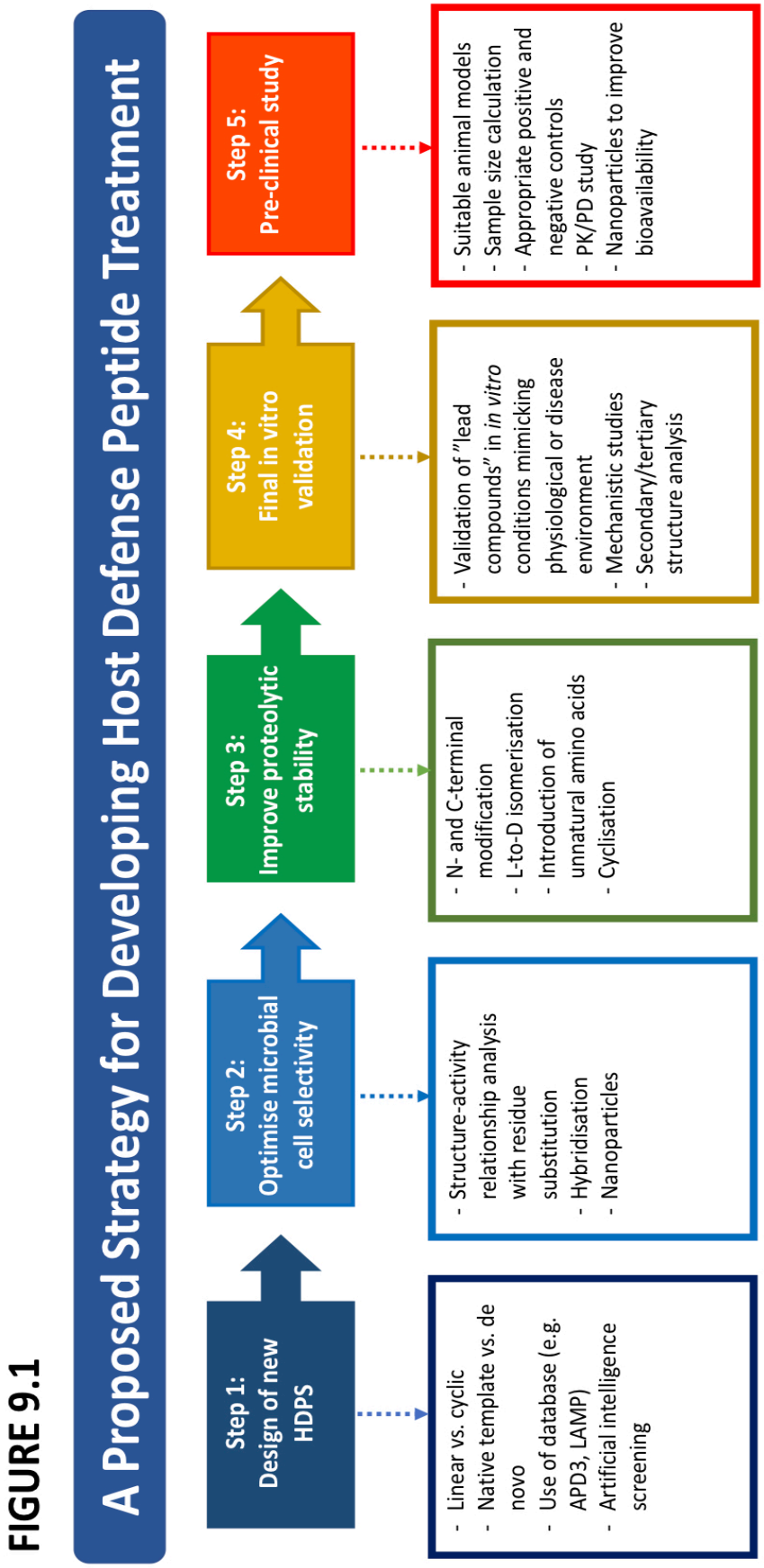
Once the efficacy and safety are optimised, the next hurdle is to overcome the issue of proteolytic degradation, which could be achieved through the strategies (either singularly or in combination) mentioned in **Figure 9.1**. However, it is noteworthy to mention that the beneficial effects of these modifications may be unique to specific HDPs. In addition, antimicrobial efficacy and/or microbial cell selectivity of the HDPs may also be affected during the modification. Subsequently, the potential lead compound should be validated in *in vitro* conditions mimicking the physiological or host disease environment. For instance, when designing HDP-based treatment for corneal infection, the designed HDPs should be tested in tear fluid or in salt of physiological concentration, which are known to affect the efficacy and stability of HDPs. The findings enable a better prediction of the *in vivo* results and help minimise the unnecessary use of animals (Mannis, 2002). Finally, well-designed pre-clinical studies need to be performed with appropriate sample size calculation and positive/negative controls, which will increase the success rate of clinical trials. For example, efficacy of the designed HDP needs to be compared with antibiotic treatment that reflects the current clinical practice for the disease of interest. Otherwise, subsequent clinical trials are likely to fail and necessary regulatory approval will not be obtained.

Ideally, it is best to optimise the previous steps before progressing to the next step. For instance, validating the potential lead compound in *in vitro* conditions mimicking physiological environment is crucial before proceeding to pre-clinical studies. Modification strategies proposed in each step may also be applicable to other steps.

For example, introduction of unnatural amino acids primarily improves the proteolytic stability but may also enhance the antimicrobial efficacy (Arias et al., 2018), and incorporation of HDPs with nanoparticles may reduce host toxicity as well as improve bioavailability. In addition, several strategies may be employed in combination to achieve the intended therapeutic effect and stability. Future work on using utilising these proposed strategies to further enhance the hybrid HDPs developed in this work will be valuable.

There are also increasing reports examining and exploiting the strategy of using peptide-antibiotic combination to counter AMR, increase the lifespan of conventional antibiotics and HDPs, as well as to reduce the undesired toxicity to host tissues (Pizzolato-Cezar et al., 2019, Lakshminarayanan et al., 2016, Mohammed et al., 2019, Lewies et al., 2019). The synergistic effect of peptide-antibiotic combination treatment is likely attributed to the different underlying mechanism of action whereby the membrane perturbation effect of peptides facilitates the passive diffusion of conventional antibiotics into the cells for intracellular targeting action (Pizzolato-Cezar et al., 2019).

Figure 9.1. Strategies for translating the therapeutic potential of HDP-based treatment.



Although HDP-based treatment has long been envisioned as a novel solution to tackle AMR, emerging evidence are suggesting that HDPs could also develop AMR, albeit with a lower risk than the conventional antibiotics (Moravej et al., 2018, Haney et al., 2019). Spohn et al. (2019) have highlighted the influence of physicochemical characteristics of HDPs, including the proportion of polar amino acids, cationicity, and hydrophobicity, on the risk of developing HDP-related AMR, thereby providing invaluable insights into the design of future HDPs. Reassuringly, cross resistance between HDPs was found to be limited to those with similar modes of action, underscoring the importance and necessity of having HDPs with different antimicrobial mechanisms within the therapeutic armamentarium of antimicrobials (Kintses et al., 2019).

With the advancement in peptide design strategy, synthesis techniques and AI technology, it is hopeful that clinical deployment of HDP-based treatment for a range of diseases will soon become a reality. However, further studies will need to be conducted to decipher the mechanism of HDP-related AMR in order to prepare for the potentially self-perpetuating vicious cycle of AMR in the future.

Upon completion of this work, I have plans to perform further research on the following areas:

1. To examine the demographic factors, clinical characteristics, risk factors, outcome and prognostic factors of fungal keratitis and Acanthamoeba keratitis in the UK.
2. To evaluate the psychosocial impact of IK on the affected patients.
3. To standardise the outcome reporting of IK in clinical trials.
4. To investigate the antimicrobial activity of the developed hybrid peptides against other types of microorganisms, including drug-resistance bacteria, fungi and Acanthamoeba.

5. To examine the pharmacokinetics/pharmacodynamics of the developed hybrid HDPs.
6. To advance the lead hybrid HDPs toward clinical trials with an aim to bring the therapy to the clinic.

9.4. Conclusion

IK is a major cause of blindness in the world. As the condition often affects one eye only, the true impact of the condition is often underestimated based on the current definition of visual impairment and blindness. A systematic approach in improving and expanding the diagnostic and therapeutic armamentarium of IK. In view of the broad-spectrum activity and low risk of developing AMR, HDP-based therapy holds great promise as a novel class of antimicrobial agent for treating local and systemic infections, including those that are affected by drug-resistant pathogens. Future in vivo work and clinical trials are required to translate the developed hybrid HDPs to clinical use.

REFERENCES

ABBOUDA, A., ABICCA, I. & ALIO, J. L. 2018. Current and future applications of photoactivated chromophore for keratitis-corneal collagen cross-linking (pack-cxl): An overview of the different treatments proposed. *Semin Ophthalmol.,* 33**,** 293-299.

ABBOUDA, A., ESTRADA, A. V., RODRIGUEZ, A. E. & ALIO, J. L. 2014. Anterior segment optical coherence tomography in evaluation of severe fungal keratitis infections treated by corneal crosslinking. *Eur J Ophthalmol,* 24**,** 320-324.

ABDULHALIM, B. E., WAGIH, M. M., GAD, A. A., BOGHDADI, G. & NAGY, R. R. 2015. Amniotic membrane graft to conjunctival flap in treatment of non-viral resistant infectious keratitis: A randomised clinical study. *Br J Ophthalmol.,* 99**,** 59-63.

ABEDIN, A., MOHAMMED, I., HOPKINSON, A. & DUA, H. S. 2008. A novel antimicrobial peptide on the ocular surface shows decreased expression in inflammation and infection. *Invest Ophthalmol Vis Sci,* 49**,** 28-33.

ABTIN, A., ECKHART, L., MILDNER, M., GRUBER, F., SCHRÖDER, J. M. & TSCHACHLER, E. 2008. Flagellin is the principal inducer of the antimicrobial peptide s100a7c (psoriasin) in human epidermal keratinocytes exposed to escherichia coli. *FASEB J.,* 22**,** 2168-76.

ACHARYA, M., FAROOQUI, J. H., SINGH, A., GANDHI, A. & MATHUR, U. 2019. *Bacterial isolates in microbial keratitis: Three-year trend analysis from north india,* Cornea, Cataract and Refractive Surgery, Dr. Shroff's Charity Eye Hospital, New Delhi, India. Laboratory Services, Dr. Shroff's Charity Eye Hospital, New Delhi, India. India.

ACHERMANN, Y., GOLDSTEIN, E. J., COENYE, T. & SHIRTLIFF, M. E. 2014. Propionibacterium acnes: From commensal to opportunistic biofilm-associated implant pathogen. *Clin Microbiol Rev,* 27**,** 419-40.

ACKERMAN, S. J., GLEICH, G. J., LOEGERING, D. A., RICHARDSON, B. A. & BUTTERWORTH, A. E. 1985. Comparative toxicity of purified human eosinophil granule cationic proteins for schistosomula of schistosoma mansoni. *Am J Trop Med Hyg.,* 34**,** 735-45.

ACKERMAN, S. J., LOEGERING, D. A., VENGE, P., OLSSON, I., HARLEY, J. B., FAUCI, A. S., et al. 1983. Distinctive cationic proteins of the human eosinophil granule: Major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J Immunol.,* 131**,** 2977-82.

AGRAWAL, N. & SINGH, S. K. 2016. Collagen cross-linking in recalcitrant corneal ulcers: A case series. *Nepal J Ophthalmol,* 8**,** 47-53.

AHMMED, A. A., TING, D. S. J. & FIGUEIREDO, F. C. 2021. Epidemiology, economic and humanistic burdens of ocular surface chemical injury: A narrative review. *Ocul Surf,* 20**,** 199-211.

AL-AQABA, M. A., DHILLON, V. K., MOHAMMED, I., SAID, D. G. & DUA, H. S. 2019. Corneal nerves in health and disease. *Prog Retin Eye Res,* 73**,** 100762.

AL-GHAFRI, A. & AL-RAISI, A. 2018. The epidemiology of nonviral microbial keratitis in a tertiary care center in muscat, oman. *Oman J Ophthalmol,* 11**,** 213-219.

ALIASHKEVICH, A., ALVAREZ, L. & CAVA, F. 2018. New insights into the mechanisms and biological roles of d-amino acids in complex eco-systems. *Front Microbiol,* 9**,** 683.

ALLEN, C. L., CLARE, G., STEWART, E. A., BRANCH, M. J., MCINTOSH, O. D., DADHWAL, M., et al. 2013. Augmented dried versus cryopreserved amniotic membrane as an ocular surface dressing. *PLoS One,* 8**,** e78441.

ALMAAYTAH, A., QAOUD, M. T., ABUALHAIJAA, A., AL-BALAS, Q. & ALZOUBI, K. H. 2018. Hybridization and antibiotic synergism as a tool for reducing the cytotoxicity of antimicrobial peptides. *Infect Drug Resist,* 11**,** 835-847.

ALTAY, Y., TAMER, S., BURCU, A. & BALTA, O. 2016. Amniotic membrane transplantation in bacterial and herpetic stromal keratitis. *Turk J Med Sci.,* 46**,** 457-462.

AMMERMANN, C., CURSIEFEN, C. & HERMANN, M. 2014. Corneal cross-linking in microbial keratitis to prevent a chaud keratoplasty: A retrospective case series. [german]. *Klinische Monatsblatter fur Augenheilkunde,* 231**,** 619-625.

ANDREU, D., UBACH, J., BOMAN, A., WAHLIN, B., WADE, D., MERRIFIELD, R. B., et al. 1992. Shortened cecropin a-melittin hybrids. Significant size reduction retains potent antibiotic activity. *FEBS Lett,* 296**,** 190-4.

ANG, M., MEHTA, J. S., SNG, C. C., HTOON, H. M. & TAN, D. T. 2012. Indications, outcomes, and risk factors for failure in tectonic keratoplasty. *Ophthalmology,* 119**,** 1311-9.

ANTHONY, C. A., PETERSON, R. A., SEWELL, D. K., POLGREEN, L. A., SIMMERING, J. E., CALLAGHAN, J. J., et al. 2018. The seasonal variability of surgical site infections in knee and hip arthroplasty. *J Arthroplasty,* 33**,** 510-514.e1.

ANTONOPLIS, A., ZANG, X., WEGNER, T., WENDER, P. A. & CEGELSKI, L. 2019. Vancomycin-arginine conjugate inhibits growth of carbapenem-resistant e. Coli and targets cell-wall synthesis. *ACS Chem Biol,* 14**,** 2065-2070.

ANWAR, H. M., EL-DANASOURY, A. M. & HASHEM, A. N. 2011. Corneal collagen crosslinking in the treatment of infectious keratitis. *Clin Ophthalmol,* 5**,** 1277-1280.

ARANCE-GIL, A., GUTIERREZ-ORTEGA, A. R., VILLA-COLLAR, C., NIETO-BONA, A., LOPES-FERREIRA, D. & GONZALEZ-MEIJOME, J. M. Corneal cross-linking for acanthamoeba keratitis in an orthokeratology patient after swimming in contaminated water. *Cont Lens Anterior Eye,* 37**,** 224-7.

ARIAS, M., PIGA, K. B., HYNDMAN, M. E. & VOGEL, H. J. 2018. Improving the activity of trp-rich antimicrobial peptides by arg/lys substitutions and changing the length of cationic residues. *Biomolecules,* 8.

ARMSTRONG, R. A. 2014. When to use the bonferroni correction. *Ophthalmic Physiol Opt,* 34**,** 502-8.

ARUNGA, S., WIAFE, G., HABTAMU, E., ONYANGO, J., GICHUHI, S., LECK, A., et al. 2019. The impact of microbial keratitis on quality of life in uganda. *BMJ Open Ophthalmol,* 4**,** e000351.

ARYA, S., AGGARWAL, M., CHANDER, J. & SOOD, S. 2008. Comparative evaluation of amniotic membrane transplantation with conventional medical treatment versus conventional medical treatment alone in suppurative keratitis. *Int J Ophthalmol Vis Sci.,* 6.

ASBELL, P. A., SANFILIPPO, C. M., PILLAR, C. M., DECORY, H. H., SAHM, D. F. & MORRIS, T. W. 2015. Antibiotic resistance among ocular pathogens in the united states: Five-year results from the antibiotic resistance monitoring in ocular microorganisms (armor) surveillance study. *JAMA Ophthalmol,* 133**,** 1445-54.

ASBELL, P. A., SANFILIPPO, C. M., SAHM, D. F. & DECORY, H. H. 2020. Trends in antibiotic resistance among ocular microorganisms in the united states from 2009 to 2018. *JAMA Ophthalmol,* 138**,** 439-450.

ASLANTURK, O. S. 2017. In vitro cytotoxicity and cell viability assays: Principles, advantages, and disadvantages. *In:* LARRAMENDY, M. L. & SOLONESKI, S. (eds.) *Genotoxicity - a predictable risk to our actual world.* IntechOpen.

ATALAY, H. T., DOGRUMAN-AL, F., SARZHANOV, F., OZMEN, M. C., TEFON, A. B., ARIBAS, Y. K., et al. 2018. Effect of riboflavin/rose bengal-mediated pack-cxl on acanthamoeba trophozoites and cysts in vitro. *Curr Eye Res,* 43**,** 1322-1325.

AUNG, T. T., YAM, J. K., LIN, S., SALLEH, S. M., GIVSKOV, M., LIU, S., et al. 2016. Biofilms of pathogenic nontuberculous mycobacteria targeted by new therapeutic approaches. *Antimicrob Agents Chemother,* 60**,** 24-35.

AUSTIN, A., LIETMAN, T. & ROSE-NUSSBAUMER, J. 2017a. Update on the management of infectious keratitis. *Ophthalmology,* 124**,** 1678-1689.

AUSTIN, A., SCHALLHORN, J., GESKE, M., MANNIS, M., LIETMAN, T. & ROSE-NUSSBAUMER, J. 2017b. Empirical treatment of bacterial keratitis: An international survey of corneal specialists. *BMJ Open Ophthalmol.,* 2.

AVRAM, S., BUIU, C., BORCAN, F. & MILAC, A. L. 2012. More effective antimicrobial mastoparan derivatives, generated by 3d-qsar-almond and computational mutagenesis. *Mol Biosyst,* 8**,** 587-94.

AYDIN, B., CUBUK, M. O., UCGUL, A., ERTOP, M., OZMEN, M. C., ATALAY, T., et al. 2020. Combined intrastromal voriconazole and amphotericin b treatment for persistent fungal keratitis. *Eye Contact Lens,* 46**,** 269-273.

BAGHERI, M., AMININASAB, M. & DATHE, M. 2018. Arginine/tryptophan-rich cyclic alpha/beta-antimicrobial peptides: The roles of hydrogen bonding and hydrophobic/hydrophilic solvent-accessible surface areas upon activity and membrane selectivity. *Chemistry,* 24**,** 14242-14253.

BAI, Y., LIU, S., JIANG, P., ZHOU, L., LI, J., TANG, C., et al. 2009. Structure-dependent charge density as a determinant of antimicrobial activity of peptide analogues of defensin. *Biochemistry.,* 48**,** 7229-39.

BAMDAD, S., MALEKHOSSEINI, H. & KHOSRAVI, A. 2015. Ultraviolet a/riboflavin collagen cross-linking for treatment of moderate bacterial corneal ulcers. *Cornea,* 34**,** 402-6.

BARUA, K., BORAH, M., DEKA, C. & KAKATI, R. 2017. Morbidity pattern and health-seeking behavior of elderly in urban slums: A cross-sectional study in assam, india. *J Family Med Prim Care,* 6**,** 345-350.

BASAIAWMOIT, P., SELVIN, S. S. T. & KORAH, S. 2018. Pack-cxl in reducing the time to heal in suppurative corneal ulcers: Observations of a pilot study from south india. *Cornea,* 37**,** 1376-1380.

BAYDA, S., ADEEL, M., TUCCINARDI, T., CORDANI, M. & RIZZOLIO, F. 2019. The history of nanoscience and nanotechnology: From chemical-physical applications to nanomedicine. *Molecules,* 25.

BECHINGER, B. 2011. Insights into the mechanisms of action of host defence peptides from biophysical and structural investigations. *J Pept Sci.,* 17**,** 306-14.

BECKER, K., HEILMANN, C. & PETERS, G. 2014. Coagulase-negative staphylococci. *Clin Microbiol Rev,* 27**,** 870-926.

BEINTEMA, J. J., WIETZES, P., WEICKMANN, J. L. & GLITZ, D. G. 1984. The amino acid sequence of human pancreatic ribonuclease. *Anal Biochem.,* 136**,** 48-64.

BERGUIGA, M., MAMELETZI, E., NICOLAS, M., RIVIER, D. & MAJO, F. 2013. Long-term follow-up of multilayer amniotic membrane transplantation (mlamt) for non-traumatic corneal perforations or deep ulcers with descemetocele. *Klinische Monatsblatter fur Augenheilkunde,* 230**,** 413-8.

BIKBOVA, G., OSHITARI, T., BABA, T., BIKBOV, M. & YAMAMOTO, S. 2018. Diabetic corneal neuropathy: Clinical perspectives. *Clin Ophthalmol,* 12**,** 981-987.

BISWARO, L. S., DA COSTA SOUSA, M. G., REZENDE, T. M. B., DIAS, S. C. & FRANCO, O. L. 2018. Antimicrobial peptides and nanotechnology, recent advances and challenges. *Front Microbiol,* 9**,** 855.

BLOMQUIST, P. H. 2006. Methicillin-resistant staphylococcus aureus infections of the eye and orbit (an american ophthalmological society thesis). *Trans Am Ophthalmol Soc,* 104**,** 322-45.

BLONDELLE, S. E., SIMPKINS, L. R., PEREZ-PAYA, E. & HOUGHTEN, R. A. 1993. Influence of tryptophan residues on melittin's hemolytic activity. *Biochim Biophys Acta,* 1202**,** 331-6.

BOMAN, H. G., WADE, D., BOMAN, I. A., WAHLIN, B. & MERRIFIELD, R. B. 1989. Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids. *FEBS Lett,* 259**,** 103-6.

BORKAR, D. S., FLEISZIG, S. M., LEONG, C., LALITHA, P., SRINIVASAN, M., GHANEKAR, A. A., et al. 2013. Association between cytotoxic and invasive pseudomonas aeruginosa and clinical outcomes in bacterial keratitis. *JAMA Ophthalmol,* 131**,** 147-53.

BOTO, A., PEREZ DE LA LASTRA, J. M. & GONZALEZ, C. C. 2018. The road from host-defense peptides to a new generation of antimicrobial drugs. *Molecules,* 23.

BOTTGER, R., HOFFMANN, R. & KNAPPE, D. 2017. Differential stability of therapeutic peptides with different proteolytic cleavage sites in blood, plasma and serum. *PLoS One,* 12**,** e0178943.

BOURCIER, T., PATTEAU, F., BORDERIE, V., BAUDRIMONT, M., RONDEAU, N., BONNEL, S., et al. 2004. [amniotic membrane transplantation for the treatment severe acanthamoeba keratitis]. *Can J Ophthalmol.,* 39**,** 621-31.

BOURCIER, T., THOMAS, F., BORDERIE, V., CHAUMEIL, C. & LAROCHE, L. 2003. Bacterial keratitis: Predisposing factors, clinical and microbiological review of 300 cases. *Br J Ophthalmol.,* 87**,** 834-8.

BOWDISH, D. M., DAVIDSON, D. J., SCOTT, M. G. & HANCOCK, R. E. 2005. Immunomodulatory activities of small host defense peptides. *Antimicrob Agents Chemother,* 49**,** 1727-32.

BRADSHAW, J. 2003. Cationic antimicrobial peptides : Issues for potential clinical use. *BioDrugs,* 17**,** 233-40.

BRAKEL, A., VOLKE, D., KRAUS, C. N., OTVOS, L. & HOFFMANN, R. 2019. Quantitation of a novel engineered anti-infective host defense peptide, arv-1502: Pharmacokinetic study of different doses in rats and dogs. *Front Chem,* 7**,** 753.

BRODERSEN, D. E., ETZERODT, M., MADSEN, P., CELIS, J. E., THØGERSEN, H. C., NYBORG, J., et al. 1998. Ef-hands at atomic resolution: The structure of human psoriasin (s100a7) solved by mad phasing. *Structure.,* 6**,** 477-89.

BRON, A. J., DE PAIVA, C. S., CHAUHAN, S. K., BONINI, S., GABISON, E. E., JAIN, S., et al. 2017. Tfos dews ii pathophysiology report. *Ocul Surf,* 15**,** 438-510.

BROOK, M., TOMLINSON, G. H., MILES, K., SMITH, R. W., ROSSI, A. G., HIEMSTRA, P. S., et al. 2016. Neutrophil-derived alpha defensins control inflammation by inhibiting macrophage mrna translation. *Proc Natl Acad Sci U S A.,* 113**,** 4350-5.

BROWN, A. C., ROSS, J., JONES, D. B., COLLIER, S. A., AYERS, T. L., HOEKSTRA, R. M., et al. 2018. Risk factors for acanthamoeba keratitis-a multistate case-control study, 2008-2011. *Eye Contact Lens,* 44**,** S173-S178.

BROWN, L. 2007. Resistance to ocular antibiotics: An overview. *Clin Exp Optom,* 90**,** 258-62.

BULET, P., STÖCKLIN, R. & MENIN, L. 2004. Anti-microbial peptides: From invertebrates to vertebrates. *Immunol Rev.,* 198**,** 169-84.

BUTLER, T. K., SPENCER, N. A., CHAN, C. C., SINGH GILHOTRA, J. & MCCLELLAN, K. 2005. Infective keratitis in older patients: A 4 year review, 1998-2002. *Br J Ophthalmol,* 89**,** 591-6.

CABRERA-AGUAS, M., KHOO, P., GEORGE, C. R. R., LAHRA, M. M. & WATSON, S. 2019. Antimicrobial resistance trends in bacterial keratitis over 5 years in sydney, australia. *Clin Exp Ophthalmol.,* 48**,** 183-91.

CAPOROSSI, A., MAZZOTTA, C., BAIOCCHI, S. & CAPOROSSI, T. 2010. Long-term results of riboflavin ultraviolet a corneal collagen cross-linking for

keratoconus in italy: The siena eye cross study. *Am J Ophthalmol,* 149**,** 585-93.

CARDOSO, M. H., CANDIDO, E. S., OSHIRO, K. G. N., REZENDE, S. B. & FRANCO, O. L. 2018. Peptides containing d-amino acids and retro-inverso peptides: General applications and special focus on antimicrobial peptides. *Peptide Applications in BIomedicine, Biotechnology and Bioengineering*.**,** 131-55.

CARDOSO, M. H., MENEGUETTI, B. T., COSTA, B. O., BUCCINI, D. F., OSHIRO, K. G. N., PREZA, S. L. E., et al. 2019a. Non-lytic antibacterial peptides that translocate through bacterial membranes to act on intracellular targets. *Int J Mol Sci,* 20.

CARDOSO, M. H., OROZCO, R. Q., REZENDE, S. B., RODRIGUES, G., OSHIRO, K. G. N., CANDIDO, E. S., et al. 2019b. Computer-aided design of antimicrobial peptides: Are we generating effective drug candidates? *Front Microbiol,* 10**,** 3097.

CARIELLO, A. J., PASSOS, R. M., YU, M. C. & HOFLING-LIMA, A. L. 2011. Microbial keratitis at a referral center in brazil. *Int Ophthalmol,* 31**,** 197-204.

CARMONA, G., RODRIGUEZ, A., JUAREZ, D., CORZO, G. & VILLEGAS, E. 2013. Improved protease stability of the antimicrobial peptide pin2 substituted with d-amino acids. *Protein J,* 32**,** 456-66.

CARNT, N., HOFFMAN, J. J., VERMA, S., HAU, S., RADFORD, C. F., MINASSIAN, D. C., et al. 2018a. Acanthamoeba keratitis: Confirmation of the uk outbreak and a prospective case-control study identifying contributing risk factors. *Br J Ophthalmol,* 102**,** 1621-1628.

CARNT, N., ROBAEI, D., MINASSIAN, D. C. & DART, J. K. G. 2018b. Acanthamoeba keratitis in 194 patients: Risk factors for bad outcomes and severe inflammatory complications. *Br J Ophthalmol,* 102**,** 1431-1435.

CASAGRANDE, M. K., FRINGS, A., KATZ, T., STEINBERG, J. & LINKE, S. J. 2014. Corneal crosslinking in pasteurella multocida-induced severe keratitis. *JCRS Online Case Reports,* 2**,** 50-53.

CASCIARO, B., MOROS, M., RIVERA-FERNANDEZ, S., BELLELLI, A., DE LA FUENTE, J. M. & MANGONI, M. L. 2017. Gold-nanoparticles coated with the antimicrobial peptide esculentin-1a(1-21)nh2 as a reliable strategy for antipseudomonal drugs. *Acta Biomater,* 47**,** 170-181.

CASTANO, G., ELNAHRY, A. G. & MADA, P. K. 2020. Fungal keratitis. *Statpearls.* Treasure Island (FL): StatPearls Publishing

CHAN, E., SNIBSON, G. R. & SULLIVAN, L. 2014. Treatment of infectious keratitis with riboflavin and ultraviolet-a irradiation. *J Cataract Refract Surg,* 40**,** 1919-1925.

CHAN, T. C. Y., CHOW, V. W. S. & JHANJI, V. 2017. Collagen cross-linking with photoactivated riboflavin (pack-cxl) for bacterial keratitis after small incision lenticule extraction (smile). *J Refract Surg,* 33**,** 278-280.

CHEN, H. C., BROWN, J. H., MORELL, J. L. & HUANG, C. M. 1988. Synthetic magainin analogues with improved antimicrobial activity. *FEBS Lett,* 236**,** 462-6.

CHEN, H. C., TAN, H. Y., HSIAO, C. H., HUANG, S. C. M., LIN, K. K. & MA, D. H. K. 2006. Amniotic membrane transplantation for persistent corneal ulcers and perforations in acute fungal keratitis. *Cornea,* 25**,** 564-572.

CHEN, J. H., MA, D. H. & TSAI, R. J. 2002. Amniotic membrane transplantation for pseudomonal keratitis with impending perforation. *Chang Gung Medical Journal,* 25**,** 144-52.

CHEN, W. L., LIN, C. T., KO, P. S., YEH, P. T., KUAN, Y. H., HU, F. R., et al. 2009. In vivo confocal microscopic findings of corneal wound healing after corneal epithelial debridement in diabetic vitrectomy. *Ophthalmology,* 116**,** 1038-47.

CHEN, X., NIYONSABA, F., USHIO, H., OKUDA, D., NAGAOKA, I., IKEDA, S., et al. 2005. Synergistic effect of antibacterial agents human beta-defensins, cathelicidin ll-37 and lysozyme against staphylococcus aureus and escherichia coli. *J Dermatol Sci.,* 40**,** 123-32.

CHEN, X., STOJANOVIC, A., EIDET, J. R. & UTHEIM, T. P. 2015. Corneal collagen cross-linking (cxl) in thin corneas. *Eye Vis (Lond),* 2**,** 15.

CHEREDDY, K. K., HER, C. H., COMUNE, M., MOIA, C., LOPES, A., PORPORATO, P. E., et al. 2014. Plga nanoparticles loaded with host defense peptide ll37 promote wound healing. *J Control Release,* 194**,** 138-47.

CHERKASOV, A., HILPERT, K., JENSSEN, H., FJELL, C. D., WALDBROOK, M., MULLALY, S. C., et al. 2009. Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic-resistant superbugs. *ACS Chem Biol,* 4**,** 65-74.

CHEW, H. F., YILDIZ, E. H., HAMMERSMITH, K. M., EAGLE, R. C., JR., RAPUANO, C. J., LAIBSON, P. R., et al. 2011. Clinical outcomes and prognostic factors associated with acanthamoeba keratitis. *Cornea.,* 30**,** 435-41.

CHIDAMBARAM, J. D., PRAJNA, N. V., LARKE, N. L., PALEPU, S., LANJEWAR, S., SHAH, M., et al. 2016. Prospective study of the diagnostic accuracy of the in vivo laser scanning confocal microscope for severe microbial keratitis. *Ophthalmology,* 123**,** 2285-2293.

CHIDAMBARAM, J. D., PRAJNA, N. V., PALEPU, S., LANJEWAR, S., SHAH, M., ELAKKIYA, S., et al. 2018a. In vivo confocal microscopy cellular features of host and organism in bacterial, fungal, and acanthamoeba keratitis. *Am J Ophthalmol,* 190**,** 24-33.

CHIDAMBARAM, J. D., VENKATESH PRAJNA, N., SRIKANTHI, P., LANJEWAR, S., SHAH, M., ELAKKIYA, S., et al. 2018b. Epidemiology, risk factors, and clinical outcomes in severe microbial keratitis in south india. *Ophthalmic Epidemiol,* 25**,** 297-305.

CHRISTOFFERSEN, R. E. 2006. Antibiotics--an investment worth making? *Nat Biotechnol.,* 24**,** 1512-4.

CHU, H. S. & HU, F. R. 2013. Non-tuberculous mycobacterial keratitis. *Clin Microbiol Infect,* 19**,** 221-6.

CLEMENS, L. E., JAYNES, J., LIM, E., KOLAR, S. S., REINS, R. Y., BAIDOURI, H., et al. 2017. Designed host defense peptides for the treatment of bacterial keratitis. *Invest Ophthalmol Vis Sci,* 58**,** 6273-6281.

CLINICAL & LABORATORY STANDARDS INSTITUTE (CLSI) 2019. Methods for dilution antimicrobial susceptibility tests for bactera that grow aerobically, 11th edition.

COLE, N., HUME, E. B., VIJAY, A. K., SANKARIDURG, P., KUMAR, N. & WILLCOX, M. D. 2010. In vivo performance of melimine as an antimicrobial coating for contact lenses in models of clare and clpu. *Invest Ophthalmol Vis Sci.,* 51**,** 390-5.

COLLIER, S. A., GRONOSTAJ, M. P., MACGURN, A. K., COPE, J. R., AWSUMB, K. L., YODER, J. S., et al. 2014. Estimated burden of keratitis--united states, 2010. *MMWR Morb Mortal Wkly Rep.,* 63**,** 1027-30.

COMUNE, M., RAI, A., CHEREDDY, K. K., PINTO, S., ADAY, S., FERREIRA, A. F., et al. 2017. Antimicrobial peptide-gold nanoscale therapeutic formulation with high skin regenerative potential. *J Control Release,* 262**,** 58-71.

COOK, S. M., GLASS, R. I., LEBARON, C. W. & HO, M. S. 1990. Global seasonality of rotavirus infections. *Bull World Health Organ,* 68**,** 171-7.

COORENS, M., SCHEENSTRA, M. R., VELDHUIZEN, E. J. & HAAGSMAN, H. P. 2017. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, tlr modulation, chemokine induction and regulation of phagocytosis. *Sci Rep.,* 7**,** 40874.

COURTRIGHT, P., LEWALLEN, S., KANJALOTI, S. & DIVALA, D. J. 1994. Traditional eye medicine use among patients with corneal disease in rural malawi. *Br J Ophthalmol,* 78**,** 810-2.

COWLAND, J. B., JOHNSEN, A. H. & BORREGAARD, N. 1995. Hcap-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Lett.,* 368**,** 173-6.

CRAIG, J. P., NICHOLS, K. K., AKPEK, E. K., CAFFERY, B., DUA, H. S., JOO, C. K., et al. 2017. Tfos dews ii definition and classification report. *Ocul Surf,* 15**,** 276-283.

CREAMER, T. P. & ROSE, G. D. 1992. Side-chain entropy opposes alpha-helix formation but rationalizes experimentally determined helix-forming propensities. *Proc Natl Acad Sci U S A,* 89**,** 5937-41.

CRISTIAN, C., MARCO, C. D. V., ARTURO, K., CLAUDIO, P., MIGUEL, S., ROLF, R., et al. 2019. Accelerated collagen cross-linking in the management of advanced acanthamoeba keratitis. *Arq Bras Oftalmol,* 82**,** 103-106.

CRUSCA, E., JR., REZENDE, A. A., MARCHETTO, R., MENDES-GIANNINI, M. J., FONTES, W., CASTRO, M. S., et al. 2011. Influence of n-terminus modifications on the biological activity, membrane interaction, and secondary structure of the antimicrobial peptide hylin-a1. *Biopolymers,* 96**,** 41-8.

CRUZ, J., FLOREZ, J., TORRES, R., URQUIZA, M., GUTIERREZ, J. A., GUZMAN, F., et al. 2017. Antimicrobial activity of a new synthetic peptide loaded in polylactic acid or poly(lactic-co-glycolic) acid nanoparticles against pseudomonas aeruginosa, escherichia coli o157:H7 and methicillin resistant staphylococcus aureus (mrsa). *Nanotechnology,* 28**,** 135102.

DAHER, K. A., SELSTED, M. E. & LEHRER, R. I. 1986. Direct inactivation of viruses by human granulocyte defensins. *J Virol.,* 60**,** 1068-74.

DAHIYA, R., KUMAR, S., KHOKRA, S. L., GUPTA, S. V., SUTARIYA, V. B., BHATIA, D., et al. 2018. Toward the synthesis and improved biopotential of an n-

methylated analog of a proline-rich cyclic tetrapeptide from marine bacteria. *Mar Drugs,* 16.

DAN, J., ZHOU, Q., ZHAI, H., CHENG, J., WAN, L., GE, C., et al. 2018. Clinical analysis of fungal keratitis in patients with and without diabetes. *PLoS One,* 13**,** e0196741.

DART, J. K., SAW, V. P. & KILVINGTON, S. 2009. Acanthamoeba keratitis: Diagnosis and treatment update 2009. *Am J Ophthalmol,* 148**,** 487-499.e2.

DARTOIS, V., SANCHEZ-QUESADA, J., CABEZAS, E., CHI, E., DUBBELDE, C., DUNN, C., et al. 2005. Systemic antibacterial activity of novel synthetic cyclic peptides. *Antimicrob Agents Chemother,* 49**,** 3302-10.

DATHE, M., NIKOLENKO, H., KLOSE, J. & BIENERT, M. 2004. Cyclization increases the antimicrobial activity and selectivity of arginine- and tryptophan-containing hexapeptides. *Biochemistry,* 43**,** 9140-50.

DATHE, M., NIKOLENKO, H., MEYER, J., BEYERMANN, M. & BIENERT, M. 2001. Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS Lett,* 501**,** 146-50.

DE ROTTH, A. 1940. Plastic repair of conjunctival defects with fetal membrane. *Arch Ophthalmol.,* 23**,** 522.

DE VIVO, M., MASETTI, M., BOTTEGONI, G. & CAVALLI, A. 2016. Role of molecular dynamics and related methods in drug discovery. *J Med Chem,* 59**,** 4035-61.

DE, Y., CHEN, Q., SCHMIDT, A. P., ANDERSON, G. M., WANG, J. M., WOOTERS, J., et al. 2000. Ll-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (fprl1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and t cells. *J Exp Med,* 192**,** 1069-74.

DEL BUEY, M. A., CRISTOBAL, J. A., CASAS, P., GONI, P., CLAVEL, A., MINGUEZ, E., et al. 2012. Evaluation of in vitro efficacy of combined riboflavin and ultraviolet a for acanthamoeba isolates. *Am J Ophthalmol,* 153**,** 399-404.

DEMIRCI, G. & OZDAMAR, A. 2013. A case of medication-resistant acanthamoeba keratitis treated by corneal crosslinking in turkey. *Case Rep Ophthalmol Med,* 2013**,** 608253.

DENNISON, S. R. & PHOENIX, D. A. 2011. Influence of c-terminal amidation on the efficacy of modelin-5. *Biochemistry,* 50**,** 1514-23.

DEORUKHKAR, S., KATIYAR, R. & SAINI, S. 2012. Epidemiological features and laboratory results of bacterial and fungal keratitis: A five-year study at a rural tertiary-care hospital in western maharashtra, india. *Singapore Med J,* 53**,** 264-7.

DESLOUCHES, B., STECKBECK, J. D., CRAIGO, J. K., DOI, Y., MIETZNER, T. A. & MONTELARO, R. C. 2013. Rational design of engineered cationic antimicrobial peptides consisting exclusively of arginine and tryptophan, and their activity against multidrug-resistant pathogens. *Antimicrob Agents Chemother,* 57**,** 2511-21.

DETHOREY, G., DARUICH, A., HAY, A., RENARD, G. & BOURGES, J. L. 2013. [severe bacterial keratitis referred to ophthalmology emergency departments: A retrospective study of 268 cases]. *J Fr Ophtalmol,* 36**,** 129-37.

DHAKHWA, K., SHARMA, M. K., BAJIMAYA, S., DWIVEDI, A. K. & RAI, S. 2012. Causative organisms in microbial keratitis, their sensitivity pattern and treatment outcome in western nepal. *Nepal J Ophthalmol,* 4**,** 119-27.

DHAWAN, S., RAO, K. & NATRAJAN, S. 2011. Complications of corneal collagen cross-linking. *J Ophthalmol,* 2011**,** 869015.

DHOPLE, V., KRUKEMEYER, A. & RAMAMOORTHY, A. 2006. The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochim Biophys Acta,* 1758**,** 1499-512.

DI ZAZZO, A., KHEIRKHAH, A., ABUD, T. B., GOYAL, S. & DANA, R. 2017. Management of high-risk corneal transplantation. *Surv Ophthalmol,* 62**,** 816-827.

DOHSE, N., WIBBELSMAN, T. D., RAPUANO, S. B., HAMMERSMITH, K. M., NAGRA, P. K., RAPUANO, C. J., et al. 2020. Microbial keratitis and clinical outcomes following penetrating and endothelial keratoplasty. *Acta Ophthalmol*.

DOMACHOWSKE, J. B., DYER, K. D., ADAMS, A. G., LETO, T. L. & ROSENBERG, H. F. 1998a. Eosinophil cationic protein/rnase 3 is another rnase a-family ribonuclease with direct antiviral activity. *Nucleic Acids Res.,* 26**,** 3358-63.

DOMACHOWSKE, J. B., DYER, K. D., BONVILLE, C. A. & ROSENBERG, H. F. 1998b. Recombinant human eosinophil-derived neurotoxin/rnase 2 functions as an effective antiviral agent against respiratory syncytial virus. *J Infect Dis.,* 177**,** 1458-64.

DONATO, R. 1999. Functional roles of s100 proteins, calcium-binding proteins of the ef-hand type. *Biochim Biophys Acta.,* 1450**,** 191-231.

DOS SANTOS CABRERA, M. P., ARCISIO-MIRANDA, M., BROGGIO COSTA, S. T., KONNO, K., RUGGIERO, J. R., PROCOPIO, J., et al. 2008. Study of the mechanism of action of anoplin, a helical antimicrobial decapeptide with ion channel-like activity, and the role of the amidated c-terminus. *J Pept Sci,* 14**,** 661-9.

DOWELL, S. F. 2001. Seasonal variation in host susceptibility and cycles of certain infectious diseases. *Emerg Infect Dis,* 7**,** 369-74.

DUA, H. S., GOMES, J. A. & SINGH, A. 1994. Corneal epithelial wound healing. *Br J Ophthalmol,* 78**,** 401-8.

DUA, H. S., GOMES, J. A. P., KING, A. J. & MAHARAJAN, V. S. 2004. The amniotic membrane in ophthalmology. *Surv Ophthalmol.,* 49**,** 51-77.

DUA, H. S., MIRI, A., ELALFY, M. S., LENCOVA, A. & SAID, D. G. 2017. Amnion-assisted conjunctival epithelial redirection in limbal stem cell grafting. *Br J Ophthalmol,* 101**,** 913-919.

DUA, H. S., OTRI, A. M., HOPKINSON, A. & MOHAMMED, I. 2014. In vitro studies on the antimicrobial peptide human beta-defensin 9 (hbd9): Signalling pathways and pathogen-related response (an american ophthalmological society thesis). *Trans Am Ophthalmol Soc,* 112**,** 50-73.

DUA, H. S., OTRI, A. M., SAID, D. G. & FARAJ, L. A. 2012. The 'up-down' sign of acute ocular surface drug toxicity. *Br J Ophthalmol,* 96**,** 1439-40.

DUA, H. S., SAID, D. G., MESSMER, E. M., ROLANDO, M., BENITEZ-DEL-CASTILLO, J. M., HOSSAIN, P. N., et al. 2018. Neurotrophic keratopathy. *Prog Retin Eye Res.,* 66**,** 107-131.

DUA, H. S., TING, D. S. J., AL SAADI, A. & SAID, D. G. 2020. Chemical eye injury: Pathophysiology, assessment and management. *Eye (Lond).* 34**,** 2001-2019.

DUA, H. S., TING, D. S. J., ALSAADI, A. & SAID, D. G. 2021. Management of limbal stem cell deficiency by amnion-assisted conjunctival epithelial redirection using vacuum-dried amniotic membrane and fibrin glue. *Br J Ophthalmol.,* doi: 10.1136/bjophthalmol-2020-318496.

DURRANT, J. D. & MCCAMMON, J. A. 2011. Molecular dynamics simulations and drug discovery. *BMC Biol,* 9**,** 71.

DUTTA, D., OZKAN, J. & WILLCOX, M. D. 2014. Biocompatibility of antimicrobial melimine lenses: Rabbit and human studies. *Optom Vis Sci.,* 91**,** 570-81.

DUTTA, D., VIJAY, A. K., KUMAR, N. & WILLCOX, M. D. 2016. Melimine-coated antimicrobial contact lenses reduce microbial keratitis in an animal model. *Invest Ophthalmol Vis Sci.,* 57**,** 5616-5624.

DUTTA, D., ZHAO, T., CHEAH, K. B., HOLMLUND, L. & WILLCOX, M. D. P. 2017. Activity of a melimine derived peptide mel4 against stenotrophomonas, delftia, elizabethkingia, burkholderia and biocompatibility as a contact lens coating. *Cont Lens Anterior Eye,* 40**,** 175-183.

ECKERT, R., HE, J., YARBROUGH, D. K., QI, F., ANDERSON, M. H. & SHI, W. 2006. Targeted killing of streptococcus mutans by a pheromone-guided "smart" antimicrobial peptide. *Antimicrob Agents Chemother,* 50**,** 3651-7.

ELEIWA, T., OZCAN, E., ABDELRAHMAN, S., SOLYMAN, O., ELHUSSEINY, A. M., YOUSSEF, G., et al. 2020. Case series of perforated keratomycosis after laser-assisted in situ keratomileusis. *Case Rep Ophthalmol Med,* 2020**,** 7237903.

ELMASSRY, A., SAID AHMED, O. I., ABDALLA, M. F. & GABALLAH, K. 2020. Ten years experience of corneal collagen cross-linking: An observational study of 6120 cases. *Eur J Ophthalmol***,** 1120672120928921.

ENGELBERG, Y. & LANDAU, M. 2020. The human ll-37(17-29) antimicrobial peptide reveals a functional supramolecular structure. *Nat Commun.,* 11**,** 3894.

ERASLAN YUSUFOGLU, E., BURCU, A., YALNIZ AKKAYA, Z. & ORNEK, F. 2013. Amniotic membrane transplantation in herpetic keratitis and bacterial keratitis. *Turk Oftalmoloiji Dergisi,* 43**,** 229-235.

ERDEM, E., HARBIYELI, I. I., BORAL, H., ILKIT, M., YAGMUR, M. & ERSOZ, R. 2018. Corneal collagen cross-linking for the management of mycotic keratitis. *Mycopathologia,* 183**,** 521-527.

ERIE, J. C., NEVITT, M. P., HODGE, D. O. & BALLARD, D. J. 1993. Incidence of ulcerative keratitis in a defined population from 1950 through 1988. *Arch Ophthalmol,* 111**,** 1665-71.

ESMAILI, E. & SHAHLAEI, M. 2015. Analysis of the flexibility and stability of the structure of magainin in a bilayer, and in aqueous and nonaqueous solutions using molecular dynamics simulations. *J Mol Model,* 21**,** 73.

ESSMANN, U., PERERA, L. & BERKOWITZ, M. L. 1995. A smooth particle mesh ewald method. *J Chem Phys.,* 103**,** 8577.

FALAGAS, M. E. & KASIAKOU, S. K. 2005. Colistin: The revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis,* 40**,** 1333-41.

FALANGA, A., NIGRO, E., DE BIASI, M. G., DANIELE, A., MORELLI, G., GALDIERO, S., et al. 2017. Cyclic peptides as novel therapeutic microbicides: Engineering of human defensin mimetics. *Molecules,* 22.

FALCIANI, C., LOZZI, L., SCALI, S., BRUNETTI, J., BRACCI, L. & PINI, A. 2014. Site-specific pegylation of an antimicrobial peptide increases resistance to pseudomonas aeruginosa elastase. *Amino Acids,* 46**,** 1403-7.

FARAJ, L. A., ELALFY, M. S., SAID, D. G. & DUA, H. S. 2014. Fine needle diathermy occlusion of corneal vessels. *Br J Ophthalmol,* 98**,** 1287-90.

FARIA, S., JOAO, I. & JORDAO, L. 2015. General overview on nontuberculous mycobacteria, biofilms, and human infection. *J Pathog,* 2015**,** 809014.

FARKOUH, A., FRIGO, P. & CZEJKA, M. 2016. Systemic side effects of eye drops: A pharmacokinetic perspective. *Clin Ophthalmol,* 10**,** 2433-2441.

FARROTTI, A., BOCCHINFUSO, G., PALLESCHI, A., ROSATO, N., SALNIKOV, E. S., VOIEVODA, N., et al. 2015. Molecular dynamics methods to predict peptide locations in membranes: Lah4 as a stringent test case. *Biochim Biophys Acta,* 1848**,** 581-92.

FERGUSON, R. & SUBRAMANIAN, V. 2018. The cellular uptake of angiogenin, an angiogenic and neurotrophic factor is through multiple pathways and largely dynamin independent. *PLoS One,* 13**,** e0193302.

FERNANDES, M., VIRA, D., DEY, M., TANZIN, T., KUMAR, N. & SHARMA, S. 2015. Comparison between polymicrobial and fungal keratitis: Clinical features, risk factors, and outcome. *Am J Ophthalmol,* 160**,** 873-881.e2.

FERNANDEZ-LOPEZ, S., KIM, H. S., CHOI, E. C., DELGADO, M., GRANJA, J. R., KHASANOV, A., et al. 2001. Antibacterial agents based on the cyclic d,l-alpha-peptide architecture. *Nature,* 412**,** 452-5.

FERRARI, G., IULIANO, L., VIGANO, M. & RAMA, P. 2013. Impending corneal perforation after collagen cross-linking for herpetic keratitis. *J Cataract Refract Surg,* 39**,** 638-641.

FERRARI, T. M., LEOZAPPA, M., LORUSSO, M., EPIFANI, E. & FERRARI, L. M. 2009. Escherichia coli keratitis treated with ultraviolet a/riboflavin corneal cross-linking: A case report. *Eur J Ophthalmol,* 19**,** 295-297.

FERREIRA, A. F., COMUNE, M., RAI, A., FERREIRA, L. & SIMOES, P. N. 2018a. Atomistic-level investigation of a ll37-conjugated gold nanoparticle by well-tempered metadynamics. *J Phys Chem B,* 122**,** 8359-8366.

FERREIRA, C. S., FIGUEIRA, L., MOREIRA-GONÇALVES, N., MOREIRA, R., TORRÃO, L. & FALCÃO-REIS, F. 2018b. Clinical and microbiological profile of bacterial microbial keratitis in a portuguese tertiary referral center-where are we in 2015? *Eye Contact Lens,* 44**,** 15-20.

FJELL, C. D., HISS, J. A., HANCOCK, R. E. & SCHNEIDER, G. 2011. Designing antimicrobial peptides: Form follows function. *Nat Rev Drug Discov.,* 11**,** 37-51.

FLANAGAN, J. L. & WILLCOX, M. D. 2009. Role of lactoferrin in the tear film. *Biochimie.,* 91**,** 35-43.

FLAXMAN, S. R., BOURNE, R. R. A., RESNIKOFF, S., ACKLAND, P., BRAITHWAITE, T., CICINELLI, M. V., et al. 2017. Global causes of blindness and distance vision impairment 1990-2020: A systematic review and meta-analysis. *Lancet Glob Health,* 5**,** e1221-e1234.

FLEISZIG, S. M. J., KROKEN, A. R., NIETO, V., GROSSER, M. R., WAN, S. J., METRUCCIO, M. M. E., et al. 2020. Contact lens-related corneal infection: Intrinsic resistance and its compromise. *Prog Retin Eye Res,* 76**,** 100804.

FLEMING, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. . *Proc R Soc B.,* 93**,** 306-17.

FONTANA, L., MORAMARCO, A., MANDARÀ, E., RUSSELLO, G. & IOVIENO, A. 2019. Interface infectious keratitis after anterior and posterior lamellar keratoplasty. Clinical features and treatment strategies. A review. *Br J Ophthalmol,* 103**,** 307-314.

FORSTER, A. J., MURFF, H. J., PETERSON, J. F., GANDHI, T. K. & BATES, D. W. 2005. Adverse drug events occurring following hospital discharge. *J Gen Intern Med,* 20**,** 317-23.

FOULSHAM, W., COCO, G., AMOUZEGAR, A., CHAUHAN, S. K. & DANA, R. 2018. When clarity is crucial: Regulating ocular surface immunity. *Trends Immunol,* 39**,** 288-301.

FOX, J. L. 2013. Antimicrobial peptides stage a comeback. *Nat Biotechnol,* 31**,** 379-82.

FOX, M. A., THWAITE, J. E., ULAETO, D. O., ATKINS, T. P. & ATKINS, H. S. 2012. Design and characterization of novel hybrid antimicrobial peptides based on cecropin a, ll-37 and magainin ii. *Peptides,* 33**,** 197-205.

FRENCH, D. D. & MARGO, C. E. 2013. Demographic patterns of ed patients diagnosed as having corneal ulcer. *Am J Emerg Med,* 31**,** 1082-5.

FRIEDBERG, D. N., STENSON, S. M., ORENSTEIN, J. M., TIERNO, P. M. & CHARLES, N. C. 1990. Microsporidial keratoconjunctivitis in acquired immunodeficiency syndrome. *Arch Ophthalmol,* 108**,** 504-8.

FU, L., JIN, P., HU, Y., LU, H. & SU, L. 2020. Kr12a6 promotes the osteogenic differentiation of human bone marrow mesenchymal stem cells via bmp/smad signaling. *Mol Med Rep,* 21**,** 61-68.

FU, R. H., LI, Y. Y. & LIU, P. 2012. Clinical observation on the curative effect of double fresh amniotic membrane covering in the treatment of fungal corneal ulcers. *Int Eye Sci.,* 12**,** 909-910.

GAIN, P., JULLIENNE, R., HE, Z., ALDOSSARY, M., ACQUART, S., COGNASSE, F., et al. 2016. Global survey of corneal transplantation and eye banking. *JAMA Ophthalmol,* 134**,** 167-73.

GALASK, R. P. & SNYDER, I. S. 1970. Antimicrobial factors in amniotic fluid. *Am J Obstet Gynecol,* 106**,** 59-65.

GANGULY, S., SALMA, K. C., KANSAKAR, I., SHARMA, M., BASTOLA, P. & PRADHAN, R. 2011. Pattern of fungal isolates in cases of corneal ulcer in the western periphery of nepal. *Nepal J Ophthalmol,* 3**,** 118-22.

GANZ, T. 2003. Defensins: Antimicrobial peptides of innate immunity. *Nat Rev Immunol.,* 3**,** 710-20.

GANZ, T., GABAYAN, V., LIAO, H. I., LIU, L., OREN, A., GRAF, T., et al. 2003. Increased inflammation in lysozyme m-deficient mice in response to micrococcus luteus and its peptidoglycan. *Blood.,* 101**,** 2388-92.

GARCIA-MONTOYA, I. A., CENDON, T. S., AREVALO-GALLEGOS, S. & RASCON-CRUZ, Q. 2012. Lactoferrin a multiple bioactive protein: An overview. *Biochim Biophys Acta.,* 1820**,** 226-36.

GARDUNO-VIEYRA, L., GONZALEZ-SANCHEZ, C. R. & HERNANDEZ-DA MOTA, S. E. 2011. Ultraviolet-a light and riboflavin therapy for acanthamoeba keratitis: A case report. *Case Rep Ophthalmol,* 2**,** 291-5.

GARG, P., DAS, S. & ROY, A. 2017a. Collagen cross-linking for microbial keratitis. *Middle East Afr J Ophthalmol,* 24**,** 18-23.

GARG, P., KALRA, P. & JOSEPH, J. 2017b. Non-contact lens related acanthamoeba keratitis. *Indian J Ophthalmol,* 65**,** 1079-1086.

GARREIS, F., GOTTSCHALT, M., SCHLORF, T., GLÄSER, R., HARDER, J., WORLITZSCH, D., et al. 2011. Expression and regulation of antimicrobial peptide psoriasin (s100a7) at the ocular surface and in the lacrimal apparatus. *Invest Ophthalmol Vis Sci.,* 52**,** 4914-22.

GAUTAM, V., CHAUDHARY, A., SINGH, S. K. & RAI, P. G. 2018. Profile of corneal ulcer in a month of harvesting season in a tertiary level eye hospital of eastern nepal. *Nepal J Ophthalmol,* 10**,** 32-38.

GAVRAS, H. & BRUNNER, H. R. 2001. Role of angiotensin and its inhibition in hypertension, ischemic heart disease, and heart failure. *Hypertension,* 37**,** 342-5.

GE, Y., MACDONALD, D. L., HOLROYD, K. J., THORNSBERRY, C., WEXLER, H. & ZASLOFF, M. 1999. In vitro antibacterial properties of pexiganan, an analog of magainin. *Antimicrob Agents Chemother,* 43**,** 782-8.

GEORNARAS, I., YOON, Y., BELK, K. E., SMITH, G. C. & SOFOS, J. N. 2007. Antimicrobial activity of epsilon-polylysine against escherichia coli o157:H7, salmonella typhimurium, and listeria monocytogenes in various food extracts. *J Food Sci,* 72**,** M330-4.

GICQUEL, J. J., BEJJANI, R. A., ELLIES, P., MERCIE, M. & DIGHIERO, P. 2007. Amniotic membrane transplantation in severe bacterial keratitis. *Cornea,* 26**,** 27-33.

GILLETTE, T. E. & ALLANSMITH, M. R. 1980. Lactoferrin in human ocular tissues. *Am J Ophthalmol.,* 90**,** 30-7.

GINOUVES, M., CARME, B., COUPPIE, P. & PREVOT, G. 2014. Comparison of tetrazolium salt assays for evaluation of drug activity against leishmania spp. *J Clin Microbiol,* 52**,** 2131-8.

GIPSON, I. K. 2007. The ocular surface: The challenge to enable and protect vision: The friedenwald lecture. *Invest Ophthalmol Vis Sci.,* 48**,** 4390; 4391-8.

GIRGIS, D. O., SLOOP, G. D., REED, J. M. & O'CALLAGHAN, R. J. 2003. A new topical model of staphylococcus corneal infection in the mouse. *Invest Ophthalmol Vis Sci,* 44**,** 1591-7.

GLÄSER, R., HARDER, J., LANGE, H., BARTELS, J., CHRISTOPHERS, E. & SCHRÖDER, J. M. 2005. Antimicrobial psoriasin (s100a7) protects human skin from escherichia coli infection. *Nat Immunol.,* 6**,** 57-64.

GONZALES, C. A., SRINIVASAN, M., WHITCHER, J. P. & SMOLIN, G. 1996. Incidence of corneal ulceration in madurai district, south india. *Ophthalmic Epidemiol,* 3**,** 159-66.

GOPAL, B. P., TITI-LARTEY, O. A., FERNANDES, P., NOUBANI, N. E., BLATHERWICK, E., SAID, D., et al. 2021. Evaluation of junior doctors' knowledge of corneal donation and the new opt-out system in england. *Postgrad Med J*.

GOPINATHAN, U., SHARMA, S., GARG, P. & RAO, G. N. 2009. Review of epidemiological features, microbiological diagnosis and treatment outcome of microbial keratitis: Experience of over a decade. *Indian J Ophthalmol,* 57**,** 273-9.

GORDON, Y. J., HUANG, L. C., ROMANOWSKI, E. G., YATES, K. A., PROSKE, R. J. & MCDERMOTT, A. M. 2005. Human cathelicidin (ll-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr Eye Res,* 30**,** 385-94.

GORSKI, M., GENIS, A., YUSHVAYEV, S., AWWAD, A. & LAZZARO, D. R. 2016. Seasonal variation in the presentation of infectious keratitis. *Eye Contact Lens,* 42**,** 295-7.

GOTTLER, L. M. & RAMAMOORTHY, A. 2009. Structure, membrane orientation, mechanism, and function of pexiganan--a highly potent antimicrobial peptide designed from magainin. *Biochim Biophys Acta,* 1788**,** 1680-6.

GRAF, M., MARDIROSSIAN, M., NGUYEN, F., SEEFELDT, A. C., GUICHARD, G., SCOCCHI, M., et al. 2017. Proline-rich antimicrobial peptides targeting protein synthesis. *Nat Prod Rep,* 34**,** 702-711.

GRAHAM, J. E., MOORE, J. E., JIRU, X., GOODALL, E. A., DOOLEY, J. S., HAYES, V. E., et al. 2007. Ocular pathogen or commensal: A pcr-based study of surface bacterial flora in normal and dry eyes. *Invest Ophthalmol Vis Sci,* 48**,** 5616-23.

GRASSI, L., MAISETTA, G., ESIN, S. & BATONI, G. 2017. Combination strategies to enhance the efficacy of antimicrobial peptides against bacterial biofilms. *Front Microbiol,* 8**,** 2409.

GREEN, M., APEL, A. & STAPLETON, F. 2008. A longitudinal study of trends in keratitis in australia. *Cornea,* 27**,** 33-9.

GREEN, M., CARNT, N., APEL, A. & STAPLETON, F. 2019a. Queensland microbial keratitis database: 2005-2015. *Br J Ophthalmol,* 103**,** 1481-1486.

GREEN, M., SARA, S., HUGHES, I., APEL, A. & STAPLETON, F. 2019b. Trends in contact lens microbial keratitis 1999 to 2015: A retrospective clinical review. *Clin Exp Ophthalmol,* 47**,** 726-732.

GRINTON, M., SANDHU, J., SHWE-TIN, A., STEEL, D. H. W. & TING, D. S. J. 2021. Incidence, characteristics, outcomes and confidence in managing posterior capsular rupture during cataract surgery in the uk: An ophthalmology trainees' perspective. *Eye (Lond).* 35**,** 1213-1220.

GRUBER, C. W., KOEHBACH, J. & MUTTENTHALER, M. 2012. Exploring bioactive peptides from natural sources for oxytocin and vasopressin drug discovery. *Future Med Chem,* 4**,** 1791-8.

GRZETIC-LENAC, R., MERLAK, M., BALOG, T., BABIC, M. B. & DEKARIS, I. 2011. Transplantation of amniotic membrane in corneal ulcers and persistant epithelial defects. *Collegium Antropologicum,* 35 Suppl 2**,** 167-9.

GUDMUNDSSON, G. H., AGERBERTH, B., ODEBERG, J., BERGMAN, T., OLSSON, B. & SALCEDO, R. 1996. The human gene fall39 and processing of the cathelin precursor to the antibacterial peptide ll-37 in granulocytes. *Eur J Biochem.,* 238**,** 325-32.

GUPTA, N., VASHIST, P., GANGER, A., TANDON, R. & GUPTA, S. K. 2018. Eye donation and eye banking in india. *Natl Med J India,* 31**,** 283-286.

GURUSAMY, K. S., GLUUD, C., NIKOLOVA, D. & DAVIDSON, B. R. 2009. Assessment of risk of bias in randomized clinical trials in surgery. *Br J Surg,* 96**,** 342-9.

HAFEZI, F. & KLING, S. 2016. Photoactivated chromophore for moderate to severe infectious keratitis as an adjunct therapy: A randomized controlled trial. *Am J Ophthalmol,* 168**,** 293-294.

HAFEZI, F. & RANDLEMAN, J. B. 2014. Pack-cxl: Defining cxl for infectious keratitis. *J Refract Surg,* 30**,** 438-9.

HAGER, T., HASENFUS, A., STACHON, T., SEITZ, B. & SZENTMARY, N. 2016. Crosslinking and corneal cryotherapy in acanthamoeba keratitis - a histological study. *Graefes Arch Clin Exp Ophthalmol,* 254**,** 149-153.

HAMET, P. & TREMBLAY, J. 2017. Artificial intelligence in medicine. *Metabolism,* 69s**,** S36-s40.

HANCOCK, R. E., HANEY, E. F. & GILL, E. E. 2016. The immunology of host defence peptides: Beyond antimicrobial activity. *Nat Rev Immunol.,* 16**,** 321-34.

HANCOCK, R. E. & LEHRER, R. 1998. Cationic peptides: A new source of antibiotics. *Trends Biotechnol.,* 16**,** 82-8.

HANCOCK, R. E. & SAHL, H. G. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol,* 24**,** 1551-7.

HANEY, E. F. & HANCOCK, R. E. 2013. Peptide design for antimicrobial and immunomodulatory applications. *Biopolymers,* 100**,** 572-83.

HANEY, E. F., MANSOUR, S. C. & HANCOCK, R. E. 2017. Antimicrobial peptides: An introduction. *Methods Mol Biol,* 1548**,** 3-22.

HANEY, E. F., STRAUS, S. K. & HANCOCK, R. E. W. 2019. Reassessing the host defense peptide landscape. *Front Chem.,* 7**,** 43.

HANSTOCK, H. G., EDWARDS, J. P. & WALSH, N. P. 2019. Tear lactoferrin and lysozyme as clinically relevant biomarkers of mucosal immune competence. *Front Immunol.,* 10**,** 1178.

HAO, L., SHAN, Q., WEI, J., MA, F. & SUN, P. 2019. Lactoferrin: Major physiological functions and applications. *Curr Protein Pept Sci.,* 20**,** 139-144.

HARDER, J., BARTELS, J., CHRISTOPHERS, E. & SCHRÖDER, J. M. 1997. A peptide antibiotic from human skin. *Nature.,* 387**,** 861.

HARRISON, S., JONES, H. E., MARTIN, R. M., LEWIS, S. J. & HIGGINS, J. P. T. 2017. The albatross plot: A novel graphical tool for presenting results of diversely reported studies in a systematic review. *Res Synth Methods,* 8**,** 281-289.

HASHEMI, H., SEYEDIAN, M. A., MIRAFTAB, M., FOTOUHI, A. & ASGARI, S. 2013. Corneal collagen cross-linking with riboflavin and ultraviolet a irradiation for keratoconus: Long-term results. *Ophthalmology,* 120**,** 1515-20.

HAYNES, R. J., TIGHE, P. J. & DUA, H. S. 1998. Innate defence of the eye by antimicrobial defensin peptides. *Lancet.,* 352**,** 451-2.

HAYNES, R. J., TIGHE, P. J. & DUA, H. S. 1999. Antimicrobial defensin peptides of the human ocular surface. *Br J Ophthalmol.,* 83**,** 737-41.

HE, M., ZHANG, H., LI, Y., WANG, G., TANG, B., ZHAO, J., et al. 2018. Cathelicidin-derived antimicrobial peptides inhibit zika virus through direct inactivation and interferon pathway. *Front Immunol.,* 9**,** 722.

HEIDARY, M., KHOSRAVI, A. D., KHOSHNOOD, S., NASIRI, M. J., SOLEIMANI, S. & GOUDARZI, M. 2018. Daptomycin. *J Antimicrob Chemother,* 73**,** 1-11.

HENRY, C. R., FLYNN, H. W., JR., MILLER, D., FORSTER, R. K. & ALFONSO, E. C. 2012. Infectious keratitis progressing to endophthalmitis: A 15-year study of microbiology, associated factors, and clinical outcomes. *Ophthalmology.,* 119**,** 2443-9.

HERNANDEZ-CAMARENA, J. C., GRAUE-HERNANDEZ, E. O., ORTIZ-CASAS, M., RAMIREZ-MIRANDA, A., NAVAS, A., PEDRO-AGUILAR, L., et al. 2015. Trends in microbiological and antibiotic sensitivity patterns in infectious keratitis: 10-year experience in mexico city. *Cornea,* 34**,** 778-85.

HESS, B., BEKKER, H., BERENDSEN, H. J. C. & FRAAIJE, J. G. E. 1998. Lincs: A linear constraint solver for molecular simulations. *J Computational Chemistry.,* 18**,** 1463-1472.

HICKS, R. P., BHONSLE, J. B., VENUGOPAL, D., KOSER, B. W. & MAGILL, A. J. 2007. De novo design of selective antibiotic peptides by incorporation of unnatural amino acids. *J Med Chem,* 50**,** 3026-36.

HIGGINS, J. P. T. & GREEN, S. 2011a. Cochrane handbook for systematic reviews of interventions. Version 5.1.0 *The Cochrane Collaboration (2011),* Available from http://handbook.cochrane.org/.

HIGGINS, J. P. T. & GREEN, S. 2011b. Cochrane handbook for systematic reviews of interventions. Version 5.1.0 (updated march 2011). The cochrane collaboration (2011). Available from http://handbook.Cochrane.Org.

HILPERT, K., VOLKMER-ENGERT, R., WALTER, T. & HANCOCK, R. E. 2005. High-throughput generation of small antibacterial peptides with improved activity. *Nat Biotechnol,* 23**,** 1008-12.

HODDENBACH, J. G., BOEKHOORN, S. S., WUBBELS, R., VREUGDENHIL, W., VAN ROOIJ, J. & GEERARDS, A. J. 2014. Clinical presentation and morbidity of contact lens-associated microbial keratitis: A retrospective study. *Graefes Arch Clin Exp Ophthalmol,* 252**,** 299-306.

HOFFMANN, S., SZENTMARY, N. & SEITZ, B. 2013. Amniotic membrane transplantation for the treatment of infectious ulcerative keratitis before elective penetrating keratoplasty. *Cornea,* 32**,** 1321-5.

HOSSAIN, P., TOURKMANI, A. K., KAZAKOS, D., JONES, M. & ANDERSON, D. 2018. Emergency corneal grafting in the uk: A 6-year analysis of the uk transplant registry. *Br J Ophthalmol.,* 102**,** 26-30.

HSIAO, C. H., SUN, C. C., YEH, L. K., MA, D. H., CHEN, P. Y., LIN, H. C., et al. 2016. Shifting trends in bacterial keratitis in taiwan: A 10-year review in a tertiary-care hospital. *Cornea,* 35**,** 313-7.

HTTP://WWW.EUCAST.ORG/CLINICAL_BREAKPOINTS/. [Accessed 15-01 2020].

HUANG, L. C., JEAN, D., PROSKE, R. J., REINS, R. Y. & MCDERMOTT, A. M. 2007a. Ocular surface expression and in vitro activity of antimicrobial peptides. *Curr Eye Res,* 32**,** 595-609.

HUANG, L. C., PETKOVA, T. D., REINS, R. Y., PROSKE, R. J. & MCDERMOTT, A. M. 2006. Multifunctional roles of human cathelicidin (ll-37) at the ocular surface. *Invest Ophthalmol Vis Sci.,* 47**,** 2369-80.

HUANG, L. C., REINS, R. Y., GALLO, R. L. & MCDERMOTT, A. M. 2007b. Cathelicidin-deficient (cnlp -/-) mice show increased susceptibility to pseudomonas aeruginosa keratitis. *Invest Ophthalmol Vis Sci.,* 48**,** 4498-508.

HUSSAIN, I., KHAN, B. S., SONI, M., IQBAL, M. & HABIBULLAH 2012. Non-viral microbial keratitis: Etiology, clinical features and visual outcome. *J Coll Physicians Surg Pak,* 22**,** 151-4.

IBRAHIM, Y. W., BOASE, D. L. & CREE, I. A. 2009. Epidemiological characteristics, predisposing factors and microbiological profiles of infectious corneal ulcers: The portsmouth corneal ulcer study. *Br J Ophthalmol,* 93**,** 1319-24.

IBRAHIM, Y. W., BOASE, D. L. & CREE, I. A. 2012. Incidence of infectious corneal ulcers, portsmouth study, uk. *J Clinic Experiment Ophthalmol.,* S6**,** 001.

IDRUS, E. A., UTTI, E. M., MATTILA, J. S. & KROOTILA, K. 2018. Photoactivated chromophore corneal cross-linking (pack-cxl) for treatment of severe keratitis. *Acta Ophthalmol*.

IGAL, V., PIKKEL IGAL, Y. S. & PIKKEL, Y. Y. 2017. Corneal cross-linking as a treatment for fungal keratitis associated with corneal melting. *Case Rep Ophthalmol,* 8**,** 148-151.

IKEDA, A., SAKIMOTO, T., SHOJI, J. & SAWA, M. 2005. Expression of alpha- and beta-defensins in human ocular surface tissue. *Jpn J Ophthalmol.,* 49**,** 73-8.

IOVIENO, A., GORE, D. M., CARNT, N. & DART, J. K. 2014. Acanthamoeba sclerokeratitis: Epidemiology, clinical features, and treatment outcomes. *Ophthalmology,* 121**,** 2340-7.

IRUDAYAM, S. J. & BERKOWITZ, M. L. 2012. Binding and reorientation of melittin in a popc bilayer: Computer simulations. *Biochim Biophys Acta,* 1818**,** 2975-81.

ISELI, H. P., THIEL, M. A., HAFEZI, F., KAMPMEIER, J. & SEILER, T. 2008. Ultraviolet a/riboflavin corneal cross-linking for infectious keratitis associated with corneal melts. *Cornea,* 27**,** 590-4.

ISLAS-RODRIGUEZ, A. E., MARCELLINI, L., ORIONI, B., BARRA, D., STELLA, L. & MANGONI, M. L. 2009. Esculentin 1-21: A linear antimicrobial peptide from frog skin with inhibitory effect on bovine mastitis-causing bacteria. *J Pept Sci,* 15**,** 607-14.

JACOB, B., PARK, I. S., BANG, J. K. & SHIN, S. Y. 2013. Short kr-12 analogs designed from human cathelicidin ll-37 possessing both antimicrobial and antiendotoxic activities without mammalian cell toxicity. *J Pept Sci,* 19**,** 700-7.

JAN, R. L., TAI, M. C., WENG, S. F., CHANG, C., WANG, J. J. & CHANG, Y. S. 2018. Risk of corneal ulcer in patients with end-stage renal disease: A retrospective large-scale cohort study. *Br J Ophthalmol,* 102**,** 868-872.

JENG, B. H., GRITZ, D. C., KUMAR, A. B., HOLSCLAW, D. S., PORCO, T. C., SMITH, S. D., et al. 2010. Epidemiology of ulcerative keratitis in northern california. *Arch Ophthalmol,* 128**,** 1022-8.

JIA, F., WANG, J., PENG, J., ZHAO, P., KONG, Z., WANG, K., et al. 2017. D-amino acid substitution enhances the stability of antimicrobial peptide polybia-cp. *Acta Biochim Biophys Sin (Shanghai),* 49**,** 916-925.

JIANG, Z., MANT, C. T., VASIL, M. & HODGES, R. S. 2018. Role of positively charged residues on the polar and non-polar faces of amphipathic alpha-helical antimicrobial peptides on specificity and selectivity for gram-negative pathogens. *Chem Biol Drug Des.,* 91**,** 75-92.

JIN, L., BAI, X., LUAN, N., YAO, H., ZHANG, Z., LIU, W., et al. 2016. A designed tryptophan- and lysine/arginine-rich antimicrobial peptide with therapeutic potential for clinical antibiotic-resistant candida albicans vaginitis. *J Med Chem,* 59**,** 1791-9.

JINQUAN, T., VORUM, H., LARSEN, C. G., MADSEN, P., RASMUSSEN, H. H., GESSER, B., et al. 1996. Psoriasin: A novel chemotactic protein. *J Invest Dermatol,* 107**,** 5-10.

JIRSOVA, K. & JONES, G. L. A. 2017. Amniotic membrane in ophthalmology: Properties, preparation, storage and indications for grafting-a review. *Cell Tissue Bank,* 18**,** 193-204.

JOHNSON, A. P., UTTLEY, A. H., WOODFORD, N. & GEORGE, R. C. 1990. Resistance to vancomycin and teicoplanin: An emerging clinical problem. *Clin Microbiol Rev,* 3**,** 280-91.

JONES, L., DOWNIE, L. E., KORB, D., BENITEZ-DEL-CASTILLO, J. M., DANA, R., DENG, S. X., et al. 2017. Tfos dews ii management and therapy report. *Ocul Surf,* 15**,** 575-628.

JORGENSEN, W. L., CHANDRASEKHAR, J., MADURA, J. D., IMPEY, R. W. & KLEIN, M. L. 1983. Comparison of simple potential functions for simulating liquid water. *J Chem Phys.,* 79**,** 926-935.

KAISERMAN, I., KAISERMAN, N., NAKAR, S. & VINKER, S. 2005. Herpetic eye disease in diabetic patients. *Ophthalmology,* 112**,** 2184-8.

KALAISELVAN, P., KONDA, N., PAMPI, N., VADDAVALLI, P. K., SHARMA, S., STAPLETON, F., et al. 2021. Effect of antimicrobial contact lenses on corneal infiltrative events: A randomized clinical trial. *Transl Vis Sci Technol,* 10**,** 32.

KALIAMURTHY, J., KALAVATHY, C. M., PARMAR, P., NELSON JESUDASAN, C. A. & THOMAS, P. A. 2013. Spectrum of bacterial keratitis at a tertiary eye care centre in india. *Biomed Res Int,* 2013**,** 181564.

KAMPSHOFF, F., WILLCOX, M. D. P. & DUTTA, D. 2019. A pilot study of the synergy between two antimicrobial peptides and two common antibiotics. *Antibiotics (Basel),* 8.

KAPOOR, G., SAIGAL, S. & ELONGAVAN, A. 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. *J Anaesthesiol Clin Pharmacol,* 33**,** 300-305.

KARANICOLAS, P. J., FARROKHYAR, F. & BHANDARI, M. 2010. Practical tips for surgical research: Blinding: Who, what, when, why, how? *Can J Surg,* 53**,** 345-8.

KASETSUWAN, N., REINPRAYOON, U. & SATITPITAKUL, V. 2016. Photoactivated chromophore for moderate to severe infectious keratitis as an adjunct therapy: A randomized controlled trial. *Am J Ophthalmol,* 165**,** 94-9.

KAYE, R., KAYE, A., SUEKE, H., NEAL, T., WINSTANLEY, C., HORSBURGH, M., et al. 2013. Recurrent bacterial keratitis. *Invest Ophthalmol Vis Sci,* 54**,** 4136-9.

KAYE, S., TUFT, S., NEAL, T., TOLE, D., LEEMING, J., FIGUEIREDO, F., et al. 2010. Bacterial susceptibility to topical antimicrobials and clinical outcome in bacterial keratitis. *Invest Ophthalmol Vis Sci,* 51**,** 362-8.

KEAY, L., EDWARDS, K., NADUVILATH, T., TAYLOR, H. R., SNIBSON, G. R., FORDE, K., et al. 2006. Microbial keratitis predisposing factors and morbidity. *Ophthalmology.,* 113**,** 109-16.

KEAY, L. J., GOWER, E. W., IOVIENO, A., OECHSLER, R. A., ALFONSO, E. C., MATOBA, A., et al. 2011. Clinical and microbiological characteristics of fungal keratitis in the united states, 2001-2007: A multicenter study. *Ophthalmology,* 118**,** 920-6.

KHALILI, M. R., JAHADI, H. R., KARIMIARIMI, M. & YASEMI, M. 2017. Corneal collagen cross-linking for treatment of bacterial and herpetic keratitis. *J Clin Diagn Res,* 11**,** NC12-NC16.

KHAN, I., SAEED, K. & KHAN, I. 2019. Nanoparticles: Properties, applications and toxicities. *Arab J Chemistry,* 12**,** 908-31.

KHAN, Y. A., KASHIWABUCHI, R. T., MARTINS, S. A., CASTRO-COMBS, J. M., KALYANI, S., STANLEY, P., et al. 2011. Riboflavin and ultraviolet light a therapy as an adjuvant treatment for medically refractive acanthamoeba keratitis: Report of 3 cases. *Ophthalmology,* 118**,** 324-331.

KHATER, M. M. 2017. Amniotic membrane graft with argon laser photocoagulation versus amniotic membrane graft with tissue debridement for treatment of mycotic keratitis. *Semin Ophthalmol.,* 32**,** 348-352.

KHEIRKHAH, A., TABATABAEI, A., ZAVAREH, M. K., KHODABANDEH, A., MOHAMMADPOUR, M. & RAJU, V. K. 2012. A controlled study of amniotic membrane transplantation for acute pseudomonas keratitis. *Can J Ophthalmol.,* 47**,** 305-11.

KHOO, P., CABRERA-AGUAS, M., ROBAEI, D., LAHRA, M. M. & WATSON, S. 2019. Microbial keratitis and ocular surface disease: A 5-year study of the microbiology, risk factors and clinical outcomes in sydney, australia. *Curr Eye Res,* 44**,** 1195-1202.

KHOO, P., CABRERA-AGUAS, M. P., NGUYEN, V., LAHRA, M. M. & WATSON, S. L. 2020. Microbial keratitis in sydney, australia: Risk factors, patient outcomes, and seasonal variation. *Graefes Arch Clin Exp Ophthalmol.,* 258**,** 1745-1755.

KHOR, H. G., CHO, I., LEE, K. & CHIENG, L. L. 2020. Spectrum of microbial keratitis encountered in the tropics. *Eye Contact Lens,* 46**,** 17-23.

KHOR, W. B., PRAJNA, V. N., GARG, P., MEHTA, J. S., XIE, L., LIU, Z., et al. 2018. The asia cornea society infectious keratitis study: A prospective multicenter study of infectious keratitis in asia. *Am J Ophthalmol,* 195**,** 161-170.

KIATSURAYANON, C., OGAWA, H. & NIYONSABA, F. 2018. The role of host defense peptide human beta-defensins in the maintenance of skin barriers. *Curr Pharm Des.,* 24**,** 1092-1099.

KIJLSTRA, A. 1990. The role of lactoferrin in the nonspecific immune response on the ocular surface. *Reg Immunol.,* 3**,** 193-7.

KIM, E. Y., RAJASEKARAN, G. & SHIN, S. Y. 2017. Ll-37-derived short antimicrobial peptide kr-12-a5 and its d-amino acid substituted analogs with cell selectivity, anti-biofilm activity, synergistic effect with conventional antibiotics, and anti-inflammatory activity. *Eur J Med Chem,* 136**,** 428-441.

KIM, H., JANG, J. H., KIM, S. C. & CHO, J. H. 2016. Enhancement of the antimicrobial activity and selectivity of gnu7 against gram-negative bacteria by fusion with lps-targeting peptide. *Peptides,* 82**,** 60-66.

KIM, H., JANG, J. H., KIM, S. C. & CHO, J. H. 2020. Development of a novel hybrid antimicrobial peptide for targeted killing of pseudomonas aeruginosa. *Eur J Med Chem,* 185**,** 111814.

KIM, J. C. & TSENG, S. C. 1995. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea,* 14**,** 473-84.

KIM, J. S., KIM, J. C., HAHN, T. W. & PARK, W. C. 2001. Amniotic membrane transplantation in infectious corneal ulcer. *Cornea,* 20**,** 720-6.

KINOSHITA, S., KOIZUMI, N., UENO, M., OKUMURA, N., IMAI, K., TANAKA, H., et al. 2018. Injection of cultured cells with a rock inhibitor for bullous keratopathy. *N Engl J Med,* 378**,** 995-1003.

KINTSES, B., JANGIR, P. K., FEKETE, G., SZAMEL, M., MEHI, O., SPOHN, R., et al. 2019. Chemical-genetic profiling reveals limited cross-resistance between antimicrobial peptides with different modes of action. *Nat Commun,* 10**,** 5731.

KNAPPE, D., PIANTAVIGNA, S., HANSEN, A., MECHLER, A., BINAS, A., NOLTE, O., et al. 2010. Oncocin (vdkppylprprpprriynr-nh2): A novel antibacterial peptide optimized against gram-negative human pathogens. *J Med Chem,* 53**,** 5240-7.

KNYAZER, B., KRAKAUER, Y., BAUMFELD, Y., LIFSHITZ, T., KLING, S. & HAFEZI, F. 2018. Accelerated corneal cross-linking with photoactivated chromophore for moderate therapy-resistant infectious keratitis. *Cornea,* 37**,** 528-531.

KOH, G. C., HAWTHORNE, G., TURNER, A. M., KUNST, H. & DEDICOAT, M. 2013. Tuberculosis incidence correlates with sunshine: An ecological 28-year time series study. *PLoS One,* 8**,** e57752.

KOLAR, S. S. & MCDERMOTT, A. M. 2011. Role of host-defence peptides in eye diseases. *Cell Mol Life Sci,* 68**,** 2201-13.

KOLAR, S. S. N., LUCA, V., BAIDOURI, H., MANNINO, G., MCDERMOTT, A. M. & MANGONI, M. L. 2015. Esculentin-1a(1-21)nh2: A frog skin-derived peptide for microbial keratitis. *Cell Mol Life Sci.,* 72**,** 617-627.

KOWALSKI, R. P., NAYYAR, S. V., ROMANOWSKI, E. G., SHANKS, R. M. Q., MAMMEN, A., DHALIWAL, D. K., et al. 2020. The prevalence of bacteria, fungi, viruses, and acanthamoeba from 3,004 cases of keratitis, endophthalmitis, and conjunctivitis. *Eye Contact Lens.,* 46**,** 265-268.

KOZOBOLIS, V., LABIRIS, G., GKIKA, M., SIDEROUDI, H., KALOGHIANNI, E., PAPADOPOULOU, D., et al. 2010. Uv-a collagen cross-linking treatment of bullous keratopathy combined with corneal ulcer. *Cornea,* 29**,** 235-8.

KRAUSE, K. M., SERIO, A. W., KANE, T. R. & CONNOLLY, L. E. 2016. Aminoglycosides: An overview. *Cold Spring Harb Perspect Med,* 6.

KRISHNAKUMARI, V. & NAGARAJ, R. 2012. Binding of peptides corresponding to the carboxy-terminal region of human-β-defensins-1-3 with model membranes investigated by isothermal titration calorimetry. *Biochim Biophys Acta,* 1818**,** 1386-94.

KUMAR, A., KHURANA, A., SHARMA, M. & CHAUHAN, L. 2019. Causative fungi and treatment outcome of dematiaceous fungal keratitis in north india. *Indian J Ophthalmol,* 67**,** 1048-1053.

KUMAR, A., PANDYA, S., KAVATHIA, G., ANTALA, S., MADAN, M. & JAVDEKAR, T. 2011. Microbial keratitis in gujarat, western india: Findings from 200 cases. *Pan Afr Med J,* 10**,** 48.

KUMAR, V., LOCKERBIE, O., KEIL, S. D., RUANE, P. H., PLATZ, M. S., MARTIN, C. B., et al. 2004. Riboflavin and uv-light based pathogen reduction: Extent and consequence of DNA damage at the molecular level. *Photochem Photobiol,* 80**,** 15-21.

KUNIMOTO, D. Y., SHARMA, S., GARG, P., GOPINATHAN, U., MILLER, D. & RAO, G. N. 2000. Corneal ulceration in the elderly in hyderabad, south india. *Br J Ophthalmol,* 84**,** 54-9.

KUZMIN, D. V., EMELIANOVA, A. A., KALASHNIKOVA, M. B., PANTELEEV, P. V. & OVCHINNIKOVA, T. V. 2017. Effect of n- and c-terminal modifications on cytotoxic properties of antimicrobial peptide tachyplesin i. *Bull Exp Biol Med,* 162**,** 754-757.

KYMIONIS, G. D., KOUROUPAKI, A. I., LIAKOPOULOS, D. A., , A., JELOVIC, I. R. & TSOULNARAS, K. I. 2016. Multiorganism, drug-resistant keratitis treated by corneal crosslinking. *Eur J Ophthalmol,* 26.

KYMIONIS, G. D., PORTALIOU, D. M., BOUZOUKIS, D. I., SUH, L. H., PALLIKARIS, A. I., MARKOMANOLAKIS, M., et al. 2007. Herpetic keratitis with iritis after corneal crosslinking with riboflavin and ultraviolet a for keratoconus. *J Cataract Refract Surg,* 33**,** 1982-4.

LABIRIS, G., GIARMOUKAKIS, A., LARIN, R., SIDEROUDI, H. & KOZOBOLIS, V. P. 2014. Corneal collagen cross-linking in a late-onset graft infectious ulcer: A case report. *J Med Case Rep,* 8.

LADRAM, A. & NICOLAS, P. 2016. Antimicrobial peptides from frog skin: Biodiversity and therapeutic promises. *Front Biosci (Landmark Ed).* 21**,** 1341-71.

LAKSHMINARAYANAN, R., TAN, W. X., AUNG, T. T., GOH, E. T., MURUGANANTHAM, N., LI, J., et al. 2016. Branched peptide, b2088, disrupts the supramolecular organization of lipopolysaccharides and sensitizes the gram-negative bacteria. *Sci Rep,* 6**,** 25905.

LAKSHMINARAYANAN, R., YE, E., YOUNG, D. J., LI, Z. & LOH, X. J. 2018. Recent advances in the development of antimicrobial nanoparticles for combating resistant pathogens. *Adv Healthc Mater,* 7**,** e1701400.

LALITHA, P., MANOHARAN, G., KARPAGAM, R., PRAJNA, N. V., SRINIVASAN, M., MASCARENHAS, J., et al. 2017. Trends in antibiotic resistance in bacterial keratitis isolates from south india. *Br J Ophthalmol.,* 101**,** 108-113.

LALITHA, P., PRAJNA, N. V., MANOHARAN, G., SRINIVASAN, M., MASCARENHAS, J., DAS, M., et al. 2015. Trends in bacterial and fungal keratitis in south india, 2002-2012. *Br J Ophthalmol,* 99**,** 192-4.

LALITHA, P., PRAJNA, N. V., OLDENBURG, C. E., SRINIVASAN, M., KRISHNAN, T., MASCARENHAS, J., et al. 2012a. Organism, minimum inhibitory concentration, and outcome in a fungal corneal ulcer clinical trial. *Cornea,* 31**,** 662-7.

LALITHA, P., SRINIVASAN, M., RAJARAMAN, R., RAVINDRAN, M., MASCARENHAS, J., PRIYA, J. L., et al. 2012b. Nocardia keratitis: Clinical course and effect of corticosteroids. *Am J Ophthalmol,* 154**,** 934-939.e1.

LANGE, C., FELTGEN, N., JUNKER, B., SCHULZE-BONSEL, K. & BACH, M. 2009. Resolving the clinical acuity categories "hand motion" and "counting fingers"

using the freiburg visual acuity test (fract). *Graefes Arch Clin Exp Ophthalmol,* 247**,** 137-42.

LARANJEIRA, P., DUQUE, M., VOJTEK, M., INÁCIO, M. J., SILVA, I., MAMEDE, A. C., et al. 2018. Amniotic membrane extract differentially regulates human peripheral blood t cell subsets, monocyte subpopulations and myeloid dendritic cells. *Cell Tissue Res,* 373**,** 459-476.

LARRICK, J. W., HIRATA, M., BALINT, R. F., LEE, J., ZHONG, J. & WRIGHT, S. C. 1995. Human cap18: A novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun,* 63**,** 1291-7.

LATA, S., SHARMA, B. K. & RAGHAVA, G. P. 2007. Analysis and prediction of antibacterial peptides. *BMC Bioinformatics,* 8**,** 263.

LAXMINARAYAN, R., DUSE, A., WATTAL, C., ZAIDI, A. K., WERTHEIM, H. F., SUMPRADIT, N., et al. 2013. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis,* 13**,** 1057-98.

LECK, A. K., THOMAS, P. A., HAGAN, M., KALIAMURTHY, J., ACKUAKU, E., JOHN, M., et al. 2002. Aetiology of suppurative corneal ulcers in ghana and south india, and epidemiology of fungal keratitis. *Br J Ophthalmol,* 86**,** 1211-5.

LECUN, Y., BENGIO, Y. & HINTON, G. 2015. Deep learning. *Nature,* 521**,** 436-44.

LEE, E. J., TRUONG, T. N., MENDOZA, M. N. & FLEISZIG, S. M. 2003. A comparison of invasive and cytotoxic pseudomonas aeruginosa strain-induced corneal disease responses to therapeutics. *Curr Eye Res,* 27**,** 289-99.

LEE, E. Y., FULAN, B. M., WONG, G. C. & FERGUSON, A. L. 2016a. Mapping membrane activity in undiscovered peptide sequence space using machine learning. *Proc Natl Acad Sci U S A,* 113**,** 13588-13593.

LEE, J. K., GOPAL, R., PARK, S. C., KO, H. S., KIM, Y., HAHM, K. S., et al. 2013. A proline-hinge alters the characteristics of the amphipathic alpha-helical amps. *PLoS One,* 8**,** e67597.

LEE, J. K., SEO, C. H., LUCHIAN, T. & PARK, Y. 2016b. Antimicrobial peptide cma3 derived from the ca-ma hybrid peptide: Antibacterial and anti-inflammatory activities with low cytotoxicity and mechanism of action in escherichia coli. *Antimicrob Agents Chemother,* 60**,** 495-506.

LEE, K. C. & ECKERT, R. L. 2007. S100a7 (psoriasin)--mechanism of antibacterial action in wounds. *J Invest Dermatol.,* 127**,** 945-57.

LEE, S. H. & TSENG, S. C. G. 1997. Amniotic membrane transplantation for persistent epithelial defects with ulceration. *Am J Ophthalmol.,* 123**,** 303-312.

LEHRER, R. I. & LU, W. 2012. Alpha-defensins in human innate immunity. *Immunol Rev,* 245**,** 84-112.

LEHRER, R. I., SZKLAREK, D., BARTON, A., GANZ, T., HAMANN, K. J. & GLEICH, G. J. 1989. Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein. *J Immunol,* 142**,** 4428-34.

LEI, H., LI, X., JING, B., XU, H. & WU, Y. 2017. Human s100a7 induces mature interleukin1α expression by rage-p38 mapk-calpain1 pathway in psoriasis. *PLoS One.,* 12**,** e0169788.

LEITCH, E. C. & WILLCOX, M. D. 1999. Elucidation of the antistaphylococcal action of lactoferrin and lysozyme. *J Med Microbiol.,* 48**,** 867-71.

LEWIES, A., DU PLESSIS, L. H. & WENTZEL, J. F. 2019. Antimicrobial peptides: The achilles' heel of antibiotic resistance? *Probiotics Antimicrob Proteins,* 11**,** 370-381.

LI, H., ANUWONGCHAROEN, N., MALIK, A. A., PRACHAYASITTIKUL, V., WIKBERG, J. E. & NANTASENAMAT, C. 2016. Roles of d-amino acids on the bioactivity of host defense peptides. *Int J Mol Sci,* 17.

LI, H., ZHANG, S., NIE, B., LONG, T., QU, X. & YUE, B. 2019a. Kr-12-a5 reverses adverse effects of lipopolysaccharides on hbmsc osteogenic differentiation by influencing bmp/smad and p38 mapk signaling pathways. *Front Pharmacol,* 10**,** 639.

LI, J., BEUERMAN, R. & VERMA, C. S. 2020a. Dissecting the molecular mechanism of colistin resistance in mcr-1 bacteria. *J Chem Inf Model,* 60**,** 4975-4984.

LI, J., BEUERMAN, R. & VERMA, C. S. 2020b. Mechanism of polyamine induced colistin resistance through electrostatic networks on bacterial outer membranes. *Biochim Biophys Acta Biomembr,* 1862**,** 183297.

LI, J., BEUERMAN, R. W. & VERMA, C. S. 2018. Molecular insights into the membrane affinities of model hydrophobes. *ACS Omega.,* 3**,** 2498-2507.

LI, J., KOH, J. J., LIU, S., LAKSHMINARAYANAN, R., VERMA, C. S. & BEUERMAN, R. W. 2017. Membrane active antimicrobial peptides: Translating mechanistic insights to design. *Front Neurosci,* 11**,** 73.

LI, J., LAKSHMINARAYANAN, R., BAI, Y., LIU, S., ZHOU, L., PERVUSHIN, K., et al. 2012. Molecular dynamics simulations of a new branched antimicrobial peptide: A comparison of force fields. *J Chem Phys,* 137**,** 215101.

LI, Q., ZHAO, G., LIN, J., WANG, Q., GUO, Y. & LIU, Y. 2014a. Clinical efficacy of the combination of lesion debridement with amniotic membrane cover and drugs for fungal keratitis. *Chinese J Experiment Ophthalmol.,* 32**,** 824-828.

LI, Q. T., ZHANG, X. F., LIU, Y. & RAN, R. J. 2014b. Clinical analysis of the amniotic membrane transplantation in the treatment of fungal corneal ulcer. *Int Eye Sci.,* 14**,** 2003-2005.

LI, S., SHENG, J., HU, J. K., YU, W., KISHIKAWA, H., HU, M. G., et al. 2013a. Ribonuclease 4 protects neuron degeneration by promoting angiogenesis, neurogenesis, and neuronal survival under stress. *Angiogenesis,* 16**,** 387-404.

LI, S., YI, G., PENG, H., LI, Z., CHEN, S., ZHONG, H., et al. 2019b. How ocular surface microbiota debuts in type 2 diabetes mellitus. *Front Cell Infect Microbiol,* 9**,** 202.

LI, X., LI, H. Y. & BAO, Y. Z. 2010. Multilayer fresh amniotic membrane transplantation for treatment of necrotizing herpes simplex keratitis. *Int J Ophthalmol,* 10**,** 794-796.

LI, X., LI, Y., HAN, H., MILLER, D. W. & WANG, G. 2006. Solution structures of human ll-37 fragments and nmr-based identification of a minimal membrane-targeting antimicrobial and anticancer region. *J Am Chem Soc,* 128**,** 5776-85.

LI, Z., JHANJI, V., TAO, X., YU, H., CHEN, W. & MU, G. 2013b. Riboflavin/ultravoilet light-mediated crosslinking for fungal keratitis. *Br J Ophthalmol,* 97**,** 669-671.

LIBERA, R. D., MELO, G. B., LIMA ADE, S., HAAPALAINEN, E. F., CRISTOVAM, P. & GOMES, J. A. 2008. Assessment of the use of cryopreserved x freeze-dried amniotic membrane (am) for reconstruction of ocular surface in rabbit model. *Arq Bras Oftalmol,* 71**,** 669-73.

LIESEGANG, T. J. 1989. Epidemiology of ocular herpes simplex. Natural history in rochester, minn, 1950 through 1982. *Arch Ophthalmol,* 107**,** 1160-5.

LIESEGANG, T. J. 2001. Herpes simplex virus epidemiology and ocular importance. *Cornea,* 20**,** 1-13.

LIM, N. C., LIM, D. K. & RAY, M. 2013. Polymicrobial versus monomicrobial keratitis: A retrospective comparative study. *Eye Contact Lens,* 39**,** 348-54.

LIN, C. C., LALITHA, P., SRINIVASAN, M., PRAJNA, N. V., MCLEOD, S. D., ACHARYA, N. R., et al. 2012. Seasonal trends of microbial keratitis in south india. *Cornea,* 31**,** 1123-7.

LIN, L., DUAN, F., YANG, Y., LOU, B., LIANG, L. & LIN, X. 2019. Nine-year analysis of isolated pathogens and antibiotic susceptibilities of microbial keratitis from a large referral eye center in southern china. *Infect Drug Resist,* 12**,** 1295-1302.

LIN, S., KOH, J. J., AUNG, T. T., LIM, F., LI, J., ZOU, H., et al. 2017. Symmetrically substituted xanthone amphiphiles combat gram-positive bacterial resistance with enhanced membrane selectivity. *J Med Chem,* 60**,** 1362-1378.

LIPSKY, B. A., HOLROYD, K. J. & ZASLOFF, M. 2008. Topical versus systemic antimicrobial therapy for treating mildly infected diabetic foot ulcers: A randomized, controlled, double-blinded, multicenter trial of pexiganan cream. *Clin Infect Dis,* 47**,** 1537-45.

LIU, C., BAYER, A., COSGROVE, S. E., DAUM, R. S., FRIDKIN, S. K., GORWITZ, R. J., et al. 2011. Clinical practice guidelines by the infectious diseases society of america for the treatment of methicillin-resistant staphylococcus aureus infections in adults and children: Executive summary. *Clin Infect Dis,* 52**,** 285-92.

LIU, J., LI, L. & LI, X. 2019. Effectiveness of cryopreserved amniotic membrane transplantation in corneal ulceration: A meta-analysis. *Cornea,* 38**,** 454-462.

LIU, S., ZHOU, L., LI, J., SURESH, A., VERMA, C., FOO, Y. H., et al. 2008. Linear analogues of human beta-defensin 3: Concepts for design of antimicrobial peptides with reduced cytotoxicity to mammalian cells. *Chembiochem.,* 9**,** 964-73.

LLOR, C. & BJERRUM, L. 2014. Antimicrobial resistance: Risk associated with antibiotic overuse and initiatives to reduce the problem. *Ther Adv Drug Saf,* 5**,** 229-41.

LOBO, A. M., AGELIDIS, A. M. & SHUKLA, D. 2019. Pathogenesis of herpes simplex keratitis: The host cell response and ocular surface sequelae to infection and inflammation. *Ocul Surf,* 17**,** 40-49.

LU, L., ARRANZ-TRULLÉN, J., PRATS-EJARQUE, G., PULIDO, D., BHAKTA, S. & BOIX, E. 2019. Human antimicrobial rnases inhibit intracellular bacterial growth and induce autophagy in mycobacteria-infected macrophages. *Front Immunol,* 10**,** 1500.

LU, L., LI, J., MOUSSAOUI, M. & BOIX, E. 2018. Immune modulation by human secreted rnases at the extracellular space. *Front Immunol,* 9**,** 1012.

LUCA, V., STRINGARO, A., COLONE, M., PINI, A. & MANGONI, M. L. 2013. Esculentin(1-21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen pseudomonas aeruginosa. *Cell Mol Life Sci.,* 70**,** 2773-86.

LUO, Y., MCLEAN, D. T., LINDEN, G. J., MCAULEY, D. F., MCMULLAN, R. & LUNDY, F. T. 2017. The naturally occurring host defense peptide, ll-37, and its truncated mimetics ke-18 and kr-12 have selected biocidal and antibiofilm activities against candida albicans, staphylococcus aureus, and escherichia coli in vitro. *Front Microbiol,* 8**,** 544.

MADSEN, P., RASMUSSEN, H. H., LEFFERS, H., HONORÉ, B., DEJGAARD, K., OLSEN, E., et al. 1991. Molecular cloning, occurrence, and expression of a novel partially secreted protein "psoriasin" that is highly up-regulated in psoriatic skin. *J Invest Dermatol,* 97**,** 701-12.

MAHARAJAN, V. S., SHANMUGANATHAN, V., CURRIE, A., HOPKINSON, A., POWELL-RICHARDS, A. & DUA, H. S. 2007. Amniotic membrane transplantation for ocular surface reconstruction: Indications and outcomes. *Clin Exp Ophthalmol,* 35**,** 140-7.

MAHARANA, P. K., SAHAY, P., SUJEETH, M., SINGHAL, D., RATHI, A., TITIYAL, J. S., et al. 2018. Microbial keratitis after accelerated corneal collagen cross-linking in keratoconus. *Cornea,* 37**,** 162-167.

MAHLAPUU, M., HAKANSSON, J., RINGSTAD, L. & BJORN, C. 2016. Antimicrobial peptides: An emerging category of therapeutic agents. *Front Cell Infect Microbiol,* 6**,** 194.

MAKADIA, H. K. & SIEGEL, S. J. 2011. Poly lactic-co-glycolic acid (plga) as biodegradable controlled drug delivery carrier. *Polymers (Basel),* 3**,** 1377-1397.

MAKDOUMI, K., MORTENSEN, J. & CRAFOORD, S. 2010. Infectious keratitis treated with corneal crosslinking. *Cornea,* 29**,** 1353-1358.

MAKDOUMI, K., MORTENSEN, J., SORKHABI, O., MALMVALL, B. E. & CRAFOORD, S. 2012. Uva-riboflavin photochemical therapy of bacterial keratitis: A pilot study. *Graefes Arch Clin Exp Ophthalmol,* 250**,** 95-102.

MALANOVIC, N., LEBER, R., SCHMUCK, M., KRIECHBAUM, M., CORDFUNKE, R. A., DRIJFHOUT, J. W., et al. 2015. Phospholipid-driven differences determine the action of the synthetic antimicrobial peptide op-145 on gram-positive bacterial and mammalian membrane model systems. *Biochim Biophys Acta,* 1848**,** 2437-47.

MALOY, W. L. & KARI, U. P. 1995. Structure-activity studies on magainins and other host defense peptides. *Biopolymers,* 37**,** 105-22.

MAMEDE, A. C., CARVALHO, M. J., ABRANTES, A. M., LARANJO, M., MAIA, C. J. & BOTELHO, M. F. 2012. Amniotic membrane: From structure and functions to clinical applications. *Cell Tissue Res,* 349**,** 447-58.

MANABE, T. & KAWASAKI, K. 2017. D-form klkllllklk-nh2 peptide exerts higher antimicrobial properties than its l-form counterpart via an association with bacterial cell wall components. *Sci Rep,* 7**,** 43384.

MANDOUR, S. S., MAREY, H. M. & FARAHAT, H. G. 2016. Resistant microbial keratitis in south nile delta, egypt: Influence of regional risk factors. *Semin Ophthalmol,* 31**,** 473-8.

MANGONI, M. L., PAPO, N., SAUGAR, J. M., BARRA, D., SHAI, Y., SIMMACO, M., et al. 2006. Effect of natural l- to d-amino acid conversion on the organization, membrane binding, and biological function of the antimicrobial peptides bombinins h. *Biochemistry,* 45**,** 4266-76.

MANIKANDAN, K. & RAMAKUMAR, S. 2004. The occurrence of c--h...O hydrogen bonds in alpha-helices and helix termini in globular proteins. *Proteins,* 56**,** 768-81.

MANNIS, M. J. 2002. The use of antimicrobial peptides in ophthalmology: An experimental study in corneal preservation and the management of bacterial keratitis. *Trans Am Ophthalmol Soc,* 100**,** 243-71.

MANSOUR, S. C., PENA, O. M. & HANCOCK, R. E. 2014. Host defense peptides: Front-line immunomodulators. *Trends Immunol.,* 35**,** 443-50.

MARDIROSSIAN, M., BARRIERE, Q., TIMCHENKO, T., MULLER, C., PACOR, S., MERGAERT, P., et al. 2018. Fragments of the nonlytic proline-rich antimicrobial peptide bac5 kill escherichia coli cells by inhibiting protein synthesis. *Antimicrob Agents Chemother,* 62.

MARDIROSSIAN, M., SOLA, R., BECKERT, B., COLLIS, D. W. P., DI STASI, A., ARMAS, F., et al. 2019a. Proline-rich peptides with improved antimicrobial activity against e. Coli, k. Pneumoniae, and a. Baumannii. *ChemMedChem,* 14**,** 2025-2033.

MARDIROSSIAN, M., SOLA, R., DEGASPERI, M. & SCOCCHI, M. 2019b. Search for shorter portions of the proline-rich antimicrobial peptide fragment bac5(1-25) that retain antimicrobial activity by blocking protein synthesis. *ChemMedChem,* 14**,** 343-348.

MARTINEZ, M. E. 2018. The calendar of epidemics: Seasonal cycles of infectious diseases. *PLoS Pathog,* 14**,** e1007327.

MARTINEZ, M. N., PAPICH, M. G. & DRUSANO, G. L. 2012. Dosing regimen matters: The importance of early intervention and rapid attainment of the pharmacokinetic/pharmacodynamic target. *Antimicrob Agents Chemother,* 56**,** 2795-805.

MARTONÁK, R., LAIO, A. & PARRINELLO, M. 2003. Predicting crystal structures: The parrinello-rahman method revisited. *Phys Rev Lett,* 90**,** 075503.

MARUJO, F. I., HIRAI, F. E., YU, M. C., HOFLING-LIMA, A. L., FREITAS, D. & SATO, E. H. 2013. [distribution of infectious keratitis in a tertiary hospital in brazil]. *Arq Bras Oftalmol,* 76**,** 370-3.

MASON, A. J., GASNIER, C., KICHLER, A., PREVOST, G., AUNIS, D., METZ-BOUTIGUE, M. H., et al. 2006. Enhanced membrane disruption and antibiotic action against pathogenic bacteria by designed histidine-rich peptides at acidic ph. *Antimicrob Agents Chemother,* 50**,** 3305-11.

MASON, A. J., MOUSSAOUI, W., ABDELRAHMAN, T., BOUKHARI, A., BERTANI, P., MARQUETTE, A., et al. 2009. Structural determinants of antimicrobial and antiplasmodial activity and selectivity in histidine-rich amphipathic cationic peptides. *J Biol Chem,* 284**,** 119-33.

MATSUZAKI, K., SUGISHITA, K., HARADA, M., FUJII, N. & MIYAJIMA, K. 1997. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of gram-negative bacteria. *Biochim Biophys Acta,* 1327**,** 119-30.

MATTILA, J. S., KORSBACK, A., KROOTILA, K. & HOLOPAINEN, J. M. 2013. Treatment of pseudomonas aeruginosa keratitis with combined corneal cross-linking and human amniotic membrane transplantation. *Acta Ophthalmol,* 91**,** e410-1.

MAYANDI, V., XI, Q., LENG GOH, E. T., KOH, S. K., JIE TOH, T. Y., BARATHI, V. A., et al. 2020. Rational substitution of ε-lysine for α-lysine enhances the cell and membrane selectivity of pore-forming melittin. *J Med Chem,* 63**,** 3522-3537.

MAZZOTTA, C., TRAVERSI, C., BAIOCCHI, S., BAGAGLIA, S., CAPOROSSI, O., VILLANO, A., et al. 2018. Corneal collagen cross-linking with riboflavin and ultraviolet a light for pediatric keratoconus: Ten-year results. *Cornea,* 37**,** 560-566.

MCCULLEY, J. P. & SHINE, W. E. 2000. Changing concepts in the diagnosis and management of blepharitis. *Cornea,* 19**,** 650-8.

MCDERMOTT, A. M. 2009. The role of antimicrobial peptides at the ocular surface. *Ophthalmic Res,* 41**,** 60-75.

MCDERMOTT, A. M. 2013. Antimicrobial compounds in tears. *Exp Eye Res,* 117**,** 53-61.

MCDONALD, E. M., RAM, F. S., PATEL, D. V. & MCGHEE, C. N. 2014. Topical antibiotics for the management of bacterial keratitis: An evidence-based review of high quality randomised controlled trials. *Br J Ophthalmol.,* 98**,** 1470-7.

MCINTOSH, R. S., CADE, J. E., AL-ABED, M., SHANMUGANATHAN, V., GUPTA, R., BHAN, A., et al. 2005. The spectrum of antimicrobial peptide expression at the ocular surface. *Invest Ophthalmol Vis Sci,* 46**,** 1379-85.

MCLEOD, S. D., KOLAHDOUZ-ISFAHANI, A., ROSTAMIAN, K., FLOWERS, C. W., LEE, P. P. & MCDONNELL, P. J. 1996. The role of smears, cultures, and antibiotic sensitivity testing in the management of suspected infectious keratitis. *Ophthalmology,* 103**,** 23-8.

MEMARIANI, H., MEMARIANI, M., SHAHIDI-DADRAS, M., NASIRI, S., AKHAVAN, M. M. & MORAVVEJ, H. 2019. Melittin: From honeybees to superbugs. *Appl Microbiol Biotechnol,* 103**,** 3265-3276.

MENCUCCI, R., PALADINI, I., MENCHINI, U., GICQUEL, J. J. & DEI, R. 2011. Inhibition of viral replication in vitro by antiviral-treated amniotic membrane. Possible use of amniotic membrane as drug-delivering tool. *Br J Ophthalmol,* 95**,** 28-31.

MENENDEZ, A. & BRETT FINLAY, B. 2007. Defensins in the immunology of bacterial infections. *Curr Opin Immunol,* 19**,** 385-91.

MIGLIOLO, L., SILVA, O. N., SILVA, P. A., COSTA, M. P., COSTA, C. R., NOLASCO, D. O., et al. 2012. Structural and functional characterization of a multifunctional alanine-rich peptide analogue from pleuronectes americanus. *PLoS One,* 7**,** e47047.

MIKA, J. T., MOISET, G., CIRAC, A. D., FELIU, L., BARDAJI, E., PLANAS, M., et al. 2011. Structural basis for the enhanced activity of cyclic antimicrobial peptides: The case of bpc194. *Biochim Biophys Acta,* 1808**,** 2197-205.

MIKUT, R., RUDEN, S., REISCHL, M., BREITLING, F., VOLKMER, R. & HILPERT, K. 2016. Improving short antimicrobial peptides despite elusive rules for activity. *Biochim Biophys Acta,* 1858**,** 1024-33.

MILES, K., CLARKE, D. J., LU, W., SIBINSKA, Z., BEAUMONT, P. E., DAVIDSON, D. J., et al. 2009. Dying and necrotic neutrophils are anti-inflammatory secondary to the release of alpha-defensins. *J Immunol.,* 183**,** 2122-32.

MISHRA, A. K., CHOI, J., MOON, E. & BAEK, K. H. 2018. Tryptophan-rich and proline-rich antimicrobial peptides. *Molecules,* 23.

MISHRA, B., LAKSHMAIAH NARAYANA, J., LUSHNIKOVA, T., WANG, X. & WANG, G. 2019. Low cationicity is important for systemic in vivo efficacy of database-derived peptides against drug-resistant gram-positive pathogens. *Proc Natl Acad Sci U S A,* 116**,** 13517-13522.

MISHRA, B., REILING, S., ZARENA, D. & WANG, G. 2017. Host defense antimicrobial peptides as antibiotics: Design and application strategies. *Curr Opin Chem Biol,* 38**,** 87-96.

MISHRA, B. & WANG, G. 2012. Ab initio design of potent anti-mrsa peptides based on database filtering technology. *J Am Chem Soc,* 134**,** 12426-9.

MISHRA, B. & WANG, G. 2017. Titanium surfaces immobilized with the major antimicrobial fragment fk-16 of human cathelicidin ll-37 are potent against multiple antibiotic-resistant bacteria. *Biofouling,* 33**,** 544-555.

MOHAMED, M. F., ABDELKHALEK, A. & SELEEM, M. N. 2016. Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular staphylococcus aureus. *Sci Rep,* 6**,** 29707.

MOHAMMED, I., MOHANTY, D., SAID, D. G., BARIK, M. R., REDDY, M. M., ALSAADI, A., et al. 2020. Antimicrobial peptides in human corneal tissue of

patients with fungal keratitis. *Br J Ophthalmol.,* doi: 10.1136/bjophthalmol-2020-316329.

MOHAMMED, I., SAID, D. G. & DUA, H. S. 2017. Human antimicrobial peptides in ocular surface defense. *Prog Retin Eye Res,* 61**,** 1-22.

MOHAMMED, I., SAID, D. G., NUBILE, M., MASTROPASQUA, L. & DUA, H. S. 2019. Cathelicidin-derived synthetic peptide improves therapeutic potential of vancomycin against pseudomonas aeruginosa. *Front Microbiol,* 10**,** 2190.

MOHAMMED, I., SULEMAN, H., OTRI, A. M., KULKARNI, B. B., CHEN, P., HOPKINSON, A., et al. 2010. Localization and gene expression of human beta-defensin 9 at the human ocular surface epithelium. *Invest Ophthalmol Vis Sci.,* 51**,** 4677-82.

MOHAMMED, I., YEUNG, A., ABEDIN, A., HOPKINSON, A. & DUA, H. S. 2011. Signalling pathways involved in ribonuclease-7 expression. *Cell Mol Life Sci,* 68**,** 1941-52.

MOHAN, S., BUDHIRAJA, I., SAXENA, A., KHAN, P. & SACHAN, S. K. 2014. Role of multilayered amniotic membrane transplantation for the treatment of resistant corneal ulcers in north india. *Int Ophthalmol.,* 34**,** 485-91.

MOHER, D., LIBERATI, A., TETZLAFF, J. & ALTMAN, D. G. 2009. The prisma group. Preferred reporting items for systematic reviews and meta-analyses: The prisma statement. *PLoS Med.,* 6**,** e1000097.

MOLINA, H. A., KIERSZENBAUM, F., HAMANN, K. J. & GLEICH, G. J. 1988. Toxic effects produced or mediated by human eosinophil granule components on trypanosoma cruzi. *Am J Trop Med Hyg,* 38**,** 327-34.

MONCLA, B. J., PRYKE, K., ROHAN, L. C. & GRAEBING, P. W. 2011. Degradation of naturally occurring and engineered antimicrobial peptides by proteases. *Adv Biosci Biotechnol,* 2**,** 404-408.

MOOKHERJEE, N., ANDERSON, M. A., HAAGSMAN, H. P. & DAVIDSON, D. J. 2020. Antimicrobial host defence peptides: Functions and clinical potential. *Nat Rev Drug Discov*.

MOOKHERJEE, N. & HANCOCK, R. E. 2007. Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections. *Cell Mol Life Sci.,* 64**,** 922-33.

MOON, J., YOON, C. H., KIM, M. K. & OH, J. Y. 2020. The incidence and outcomes of recurrence of infection after therapeutic penetrating keratoplasty for medically-uncontrolled infectious keratitis. *J Clin Med,* 9.

MORAVEJ, H., MORAVEJ, Z., YAZDANPARAST, M., HEIAT, M., MIRHOSSEINI, A., MOOSAZADEH MOGHADDAM, M., et al. 2018. Antimicrobial peptides: Features, action, and their resistance mechanisms in bacteria. *Microb Drug Resist,* 24**,** 747-767.

MOREN, H., MALMSJO, M., MORTENSEN, J. & OHRSTROM, A. 2010. Riboflavin and ultraviolet a collagen crosslinking of the cornea for the treatment of keratitis. *Cornea,* 29**,** 102-104.

MOSHIRFAR, M., SOMANI, S. N., SHMUNES, K. M., ESPANDAR, L., GOKHALE, N. S., RONQUILLO, Y. C., et al. 2020. A narrative review of microsporidial infections of the cornea. *Ophthalmol Ther,* 9**,** 265-278.

MULLER, L., THIEL, M. A., KIPFER-KAUER, A. I. & KAUFMANN, C. 2012. Corneal cross-linking as supplementary treatment option in melting keratitis: A case series. *Klinische Monatsblatter fur Augenheilkunde,* 229**,** 411-415.

MUNITA, J. M. & ARIAS, C. A. 2016. Mechanisms of antibiotic resistance. *Microbiol Spectr,* 4.

MURA, M., WANG, J., ZHOU, Y., PINNA, M., ZVELINDOVSKY, A. V., DENNISON, S. R., et al. 2016. The effect of amidation on the behaviour of antimicrobial peptides. *Eur Biophys J,* 45**,** 195-207.

MWANGI, J., YIN, Y., WANG, G., YANG, M., LI, Y., ZHANG, Z., et al. 2019. The antimicrobial peptide zy4 combats multidrug-resistant pseudomonas aeruginosa and acinetobacter baumannii infection. *Proc Natl Acad Sci U S A*.

NAEEM, M., AHMAD, M., KHAN, H. M. & KHAN, M. N. 2014. Comparative evaluation of conventional medical treatment alone versus conventional medical treatment with amniotic membrane transplantation in infective corneal ulcer. *J Postgrad Med Institute.,* 28**,** 206-210.

NAKAMURA, T., YOSHITANI, M., RIGBY, H., FULLWOOD, N. J., ITO, W., INATOMI, T., et al. 2004. Sterilized, freeze-dried amniotic membrane: A useful substrate for ocular surface reconstruction. *Invest Ophthalmol Vis Sci,* 45**,** 93-9.

NARAYANA, J. L., MISHRA, B., LUSHNIKOVA, T., GOLLA, R. M. & WANG, G. 2019a. Modulation of antimicrobial potency of human cathelicidin peptides against the eskape pathogens and in vivo efficacy in a murine catheter-associated biofilm model. *Biochim Biophys Acta Biomembr,* 1861**,** 1592-1602.

NARAYANA, S., KRISHNAN, T., RAMAKRISHNAN, S., SAMANTARAY, P. P., AUSTIN, A., PICKEL, J., et al. 2019b. Mycotic antimicrobial localized injection: A randomized clinical trial evaluating intrastromal injection of voriconazole. *Ophthalmology,* 126**,** 1084-1089.

NASEEM, I., AHMAD, M. & HADI, S. M. 1988. Effect of alkylated and intercalated DNA on the generation of superoxide anion by riboflavin. *Biosci Rep,* 8**,** 485-92.

NATAN, M. & BANIN, E. 2017. From nano to micro: Using nanotechnology to combat microorganisms and their multidrug resistance. *FEMS Microbiol Rev,* 41**,** 302-322.

NELL, M. J., TJABRINGA, G. S., WAFELMAN, A. R., VERRIJK, R., HIEMSTRA, P. S., DRIJFHOUT, J. W., et al. 2006. Development of novel ll-37 derived antimicrobial peptides with lps and lta neutralizing and antimicrobial activities for therapeutic application. *Peptides,* 27**,** 649-60.

NGUYEN, K. T., LE CLAIR, S. V., YE, S. & CHEN, Z. 2009. Molecular interactions between magainin 2 and model membranes in situ. *J Phys Chem B,* 113**,** 12358-63.

NGUYEN, L. T., HANEY, E. F. & VOGEL, H. J. 2011. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol,* 29**,** 464-72.

NI, N., NAM, E. M., HAMMERSMITH, K. M., NAGRA, P. K., AZARI, A. A., LEIBY, B. E., et al. 2015. Seasonal, geographic, and antimicrobial resistance patterns in microbial keratitis: 4-year experience in eastern pennsylvania. *Cornea.,* 34**,** 296-302.

NICHOLAS, M. P. & MYSORE, N. 2020. Corneal neovascularization. *Exp Eye Res***,** 108363.

NIZEYIMANA, H., ZHOU, D. D., LIU, X. F., PAN, X. T., LIU, C., LU, C. W., et al. 2017. Clinical efficacy of conjunctival flap surgery in the treatment of refractory fungal keratitis. *Exp Ther Med,* 14**,** 1109-1113.

NOSE, S. & KLEIN, M. L. 1983. Constant pressure molecular dynamics for molecular systems. *Mol Phys.,* 50**,** 1055-1076.

NOTARA, M., SHORTT, A. J., O'CALLAGHAN, A. R. & DANIELS, J. T. 2013. The impact of age on the physical and cellular properties of the human limbal stem cell niche. *Age (Dordr),* 35**,** 289-300.

NUBILE, M., DUA, H. S., LANZINI, M., CIANCAGLINI, M., CALIENNO, R., SAID, D. G., et al. 2011. In vivo analysis of stromal integration of multilayer amniotic membrane transplantation in corneal ulcers. *American Journal of Ophthalmology,* 151**,** 809-822.e1.

O'NEIL, K. T. & DEGRADO, W. F. 1990. A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. *Science,* 250**,** 646-51.

O'NEILL, J. 2016. Tackling drug-resistant infections globally: Final report and recommendations. *Review on Antimicrobial Resistance.***,** 1-81.

OFLAZ, A. B., BOZKURT, B., KAMIS, U. & KOKTEKIR, B. E. 2017. Corneal collagen crosslinking treatment in a case with pneumococcal keratitis. *Turk Oftalmoloiji Dergisi,* 47**,** 161-164.

OLADIGBOLU, K., RAFINDADI, A., ABAH, E. & SAMAILA, E. 2013. Corneal ulcers in a tertiary hospital in northern nigeria. *Ann Afr Med,* 12**,** 165-70.

OLDENBURG, C. E., LALITHA, P., SRINIVASAN, M., RAJARAMAN, R., RAVINDRAN, M., MASCARENHAS, J., et al. 2013. Emerging moxifloxacin resistance in pseudomonas aeruginosa keratitis isolates in south india. *Ophthalmic Epidemiol,* 20**,** 155-8.

OLIVA, R., CHINO, M., PANE, K., PISTORIO, V., DE SANTIS, A., PIZZO, E., et al. 2018. Exploring the role of unnatural amino acids in antimicrobial peptides. *Sci Rep,* 8**,** 8888.

ONG, P. Y., OHTAKE, T., BRANDT, C., STRICKLAND, I., BOGUNIEWICZ, M., GANZ, T., et al. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med.,* 347**,** 1151-60.

ORLANS, H. O., HORNBY, S. J. & BOWLER, I. C. 2011. In vitro antibiotic susceptibility patterns of bacterial keratitis isolates in oxford, uk: A 10-year review. *Eye (Lond),* 25**,** 489-93.

OTRI, A. M., FARES, U., AL-AQABA, M. A. & DUA, H. S. 2012a. Corneal densitometry as an indicator of corneal health. *Ophthalmology,* 119**,** 501-8.

OTRI, A. M., FARES, U., AL-AQABA, M. A., MIRI, A., FARAJ, L. A., SAID, D. G., et al. 2013. Profile of sight-threatening infectious keratitis: A prospective study. *Acta Ophthalmol.,* 91**,** 643-51.

OTRI, A. M., MOHAMMED, I., ABEDIN, A., CAO, Z., HOPKINSON, A., PANJWANI, N., et al. 2010. Antimicrobial peptides expression by ocular surface cells in response to acanthamoeba castellanii: An in vitro study. *Br J Ophthalmol.,* 94**,** 1523-7.

OTRI, A. M., MOHAMMED, I., AL-AQABA, M. A., FARES, U., PENG, C., HOPKINSON, A., et al. 2012b. Variable expression of human beta defensins 3 and 9 at the human ocular surface in infectious keratitis. *Invest Ophthalmol Vis Sci,* 53**,** 757-61.

OUZZANI, M., HAMMADY, H., FEDOROWICZ, Z. & ELMAGARMID, A. 2016. Rayyan-a web and mobile app for systematic reviews. *Syst Rev,* 5**,** 210.

PAN, X. J., JIANG, T., ZHU, H., LIU, P. P., ZHOU, Z. Y. & MAO, A. J. 2016. Corneal infection in shandong peninsula of china: A 10-year retrospective study on 578 cases. *Int J Ophthalmol,* 9**,** 53-7.

PANDA, A., KRISHNA, S. N. & KUMAR, S. 2012. Photo-activated riboflavin therapy of refractory corneal ulcers. *Cornea,* 31**,** 1210-1213.

PAPAIOANNOU, L., MILIGKOS, M. & PAPATHANASSIOU, M. 2016. Corneal collagen cross-linking for infectious keratitis: A systematic review and meta-analysis. *Cornea,* 35**,** 62-71.

PARIS FDOS, S., GONÇALVES, E. D., CAMPOS, M. S., SATO, E. H., DUA, H. S. & GOMES, J. 2013. Amniotic membrane transplantation versus anterior stromal puncture in bullous keratopathy: A comparative study. *Br J Ophthalmol,* 97**,** 980-4.

PARMAR, P., SALMAN, A., KALAVATHY, C. M., KALIAMURTHY, J., THOMAS, P. A. & JESUDASAN, C. A. 2006. Microbial keratitis at extremes of age. *Cornea,* 25**,** 153-8.

PASSILONGO, M., PEDROTTI, E., TALLI, P. M., COMACCHIO, F., FASOLO, A., BONACCI, E., et al. Accelerated corneal crosslinking to treat acanthamoeba and fusarium coinfection of the cornea. *JCRS Online Case Reports,* 6**,** 19-21.

PATRA, J. K., DAS, G., FRACETO, L. F., CAMPOS, E. V. R., RODRIGUEZ-TORRES, M. D. P., ACOSTA-TORRES, L. S., et al. 2018. Nano based drug delivery systems: Recent developments and future prospects. *J Nanobiotechnology,* 16**,** 71.

PAZGIER, M., HOOVER, D. M., YANG, D., LU, W. & LUBKOWSKI, J. 2006. Human beta-defensins. *Cell Mol Life Sci.,* 63**,** 1294-313.

PENG, M. Y., CEVALLOS, V., MCLEOD, S. D., LIETMAN, T. M. & ROSE-NUSSBAUMER, J. 2018. Bacterial keratitis: Isolated organisms and antibiotic resistance patterns in san francisco. *Cornea,* 37**,** 84-87.

PIZZOLATO-CEZAR, L. R., OKUDA-SHINAGAWA, N. M. & MACHINI, M. T. 2019. Combinatory therapy antimicrobial peptide-antibiotic to minimize the ongoing rise of resistance. *Front Microbiol,* 10**,** 1703.

PLETZER, D., COLEMAN, S. R. & HANCOCK, R. E. 2016. Anti-biofilm peptides as a new weapon in antimicrobial warfare. *Curr Opin Microbiol.,* 33**,** 35-40.

POLITIS, M., WAJNSZTAJN, D., ROSIN, B., BLOCK, C. & SOLOMON, A. 2016. Trends of bacterial keratitis culture isolates in jerusalem; a 13- years analysis. *PLoS One,* 11**,** e0165223.

PONTES, F. J., RUSU, V. H., SOARES, T. A. & LINS, R. D. 2012. The effect of temperature, cations, and number of acyl chains on the lamellar to non-lamellar transition in lipid-a membranes: A microscopic view. *J Chem Theory Comput,* 8**,** 3830-8.

PRAJNA, N. V., KRISHNAN, T., RAJARAMAN, R., PATEL, S., SHAH, R., SRINIVASAN, M., et al. 2017. Adjunctive oral voriconazole treatment of fusarium keratitis: A secondary analysis from the mycotic ulcer treatment trial ii. *JAMA Ophthalmol,* 135**,** 520-525.

PRAJNA, N. V., LALITHA, P., RAJARAMAN, R., KRISHNAN, T., RAGHAVAN, A., SRINIVASAN, M., et al. 2016. Changing azole resistance: A secondary analysis of the mutt i randomized clinical trial. *JAMA Ophthalmol,* 134**,** 693-6.

PRAJNA, N. V., RADHAKRISHNAN, N., LALITHA, P., AUSTIN, A., RAY, K. J., KEENAN, J. D., et al. 2020. Cross-linking-assisted infection reduction: A randomized clinical trial evaluating the effect of adjuvant cross-linking on outcomes in fungal keratitis. *Ophthalmology,* 127**,** 159-166.

PRESTINACI, F., PEZZOTTI, P. & PANTOSTI, A. 2015. Antimicrobial resistance: A global multifaceted phenomenon. *Pathog Glob Health,* 109**,** 309-18.

PRICE, M. O., TENKMAN, L. R., SCHRIER, A., FAIRCHILD, K. M., TROKEL, S. L. & PRICE JR, F. W. 2012. Photoactivated riboflavin treatment of infectious keratitis using collagen cross-linking technology. *J Refract Surg,* 28**,** 706-713.

PURWIN, M., MARKOWSKA, A., BRUZGO, I., RUSAK, T., SURAZYNSKI, A., JAWOROWSKA, U., et al. 2017. Peptides with 6-aminohexanoic acid: Synthesis and evaluation as plasmin inhibitors. *Int J Pept Res Ther,* 23**,** 235-245.

PUTSEP, K., BRANDEN, C. I., BOMAN, H. G. & NORMARK, S. 1999. Antibacterial peptide from h. Pylori. *Nature,* 398**,** 671-2.

QVIT, N., RUBIN, S. J. S., URBAN, T. J., MOCHLY-ROSEN, D. & GROSS, E. R. 2017. Peptidomimetic therapeutics: Scientific approaches and opportunities. *Drug Discov Today,* 22**,** 454-462.

RAGLAND, S. A. & CRISS, A. K. 2017. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathog,* 13**,** e1006512.

RAHEEM, N. & STRAUS, S. K. 2019. Mechanisms of action for antimicrobial peptides with antibacterial and antibiofilm functions. *Front Microbiol,* 10**,** 2866.

RAINES, R. T. 1998. Ribonuclease a. *Chem Rev,* 98**,** 1045-1066.

RAJASEKARAN, G., KIM, E. Y. & SHIN, S. Y. 2017. Ll-37-derived membrane-active fk-13 analogs possessing cell selectivity, anti-biofilm activity and synergy with chloramphenicol and anti-inflammatory activity. *Biochim Biophys Acta Biomembr,* 1859**,** 722-733.

RAMIREZ-RICO, G., MARTINEZ-CASTILLO, M., DE LA GARZA, M., SHIBAYAMA, M. & SERRANO-LUNA, J. 2015. Acanthamoeba castellanii proteases are capable of degrading iron-binding proteins as a possible mechanism of pathogenicity. *J Eukaryot Microbiol.,* 62**,** 614-22.

RAMONA, B. I., CATALINA, C., ANDREI, M., DACIANA, S. & CALIN, T. 2016. Collagen crosslinking in the management of microbial keratitis. *Rom J Ophthalmol,* 60**,** 28-30.

RAMOS, R., SILVA, J. P., RODRIGUES, A. C., COSTA, R., GUARDAO, L., SCHMITT, F., et al. 2011. Wound healing activity of the human antimicrobial peptide ll37. *Peptides,* 32**,** 1469-76.

RAMPAT, R., DESHMUKH, R., CHEN, X., TING, D. S. W., SAID, D. G., DUA, H. S., et al. 2021. Artificial intelligence in cornea, refractive surgery, and cataract: Basic principles, clinical applications, and future directions. *Asia Pac J Ophthalmol (Phila),* 10**,** 268-281.

RANDLEMAN, J. B. & SHAH, R. D. 2012. Lasik interface complications: Etiology, management, and outcomes. *J Refract Surg,* 28**,** 575-86.

RAO, M. Q., HUANG, S., WANG, X. Y. & XU, L. 2012. Amniotic membrane transplantation and continuous ring lock shaped suture for the treatment of fungal corneal ulcer. *Int Eye Sci.,* 12**,** 1375-1376.

RAUTARAYA, B., SHARMA, S., KAR, S., DAS, S. & SAHU, S. K. 2011. Diagnosis and treatment outcome of mycotic keratitis at a tertiary eye care center in eastern india. *BMC Ophthalmol,* 11**,** 39.

RAY, K. J., PRAJNA, L., SRINIVASAN, M., GEETHA, M., KARPAGAM, R., GLIDDEN, D., et al. 2013. Fluoroquinolone treatment and susceptibility of isolates from bacterial keratitis. *JAMA Ophthalmol,* 131**,** 310-3.

REDFERN, R. L. & MCDERMOTT, A. M. 2010. Toll-like receptors in ocular surface disease. *Exp Eye Res.,* 90**,** 679-87.

REDFERN, R. L., REINS, R. Y. & MCDERMOTT, A. M. 2011. Toll-like receptor activation modulates antimicrobial peptide expression by ocular surface cells. *Exp Eye Res.,* 92**,** 209-20.

REE, R., VARLAND, S. & ARNESEN, T. 2018. Spotlight on protein n-terminal acetylation. *Exp Mol Med,* 50**,** 90.

REN, S. X., CHENG, A. S., TO, K. F., TONG, J. H., LI, M. S., SHEN, J., et al. 2012. Host immune defense peptide ll-37 activates caspase-independent apoptosis and suppresses colon cancer. *Cancer Res.,* 72**,** 6512-23.

REN, S. X., SHEN, J., CHENG, A. S., LU, L., CHAN, R. L., LI, Z. J., et al. 2013. Fk-16 derived from the anticancer peptide ll-37 induces caspase-independent apoptosis and autophagic cell death in colon cancer cells. *PLoS One.,* 8**,** e63641.

RESHMA, V. G., SYAMA, S., SRUTHI, S., RESHMA, S. C., REMYA, N. S. & MOHANAN, P. V. 2017. Engineered nanoparticles with antimicrobial property. *Curr Drug Metab,* 18**,** 1040-1054.

REVIEW MANAGER (REVMAN) VERSION 5.4.1 2020. Copenhagan: The Nordic Cochrane Centre, The Cochrane Collaboration.

RICHOZ, O., GATZIOUFAS, Z., FRANCOIS, P., SCHRENZEL, J. & HAFEZI, F. 2013. Impact of fluorescein on the antimicrobial efficacy of photoactivated riboflavin in corneal collagen cross-linking. *J Refract Surg,* 29**,** 842-5.

RIEDL, S., ZWEYTICK, D. & LOHNER, K. 2011. Membrane-active host defense peptides--challenges and perspectives for the development of novel anticancer drugs. *Chem Phys Lipids.,* 164**,** 766-81.

RIOOL, M., DE BREIJ, A., DRIJFHOUT, J. W., NIBBERING, P. H. & ZAAT, S. A. J. 2017. Antimicrobial peptides in biomedical device manufacturing. *Front Chem,* 5**,** 63.

ROBAEI, D., CARNT, N. & WATSON, S. 2016. Established and emerging ancillary techniques in management of microbial keratitis: A review. *Br J Ophthalmol.,* 100**,** 1163-70.

ROMEO, D., SKERLAVAJ, B., BOLOGNESI, M. & GENNARO, R. 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. *J Biol Chem.,* 263**,** 9573-5.

ROSENBERG, H. F. 2008. Rnase a ribonucleases and host defense: An evolving story. *J Leukoc Biol,* 83**,** 1079-87.

ROSETTA, P., VINCIGUERRA, R., ROMANO, M. R. & VINCIGUERRA, P. 2013. Corneal collagen cross-linking window absorption. *Cornea,* 32**,** 550-554.

SAGERFORS, S., EJDERVIK-LINDBLAD, B. & SÖDERQUIST, B. 2019. Infectious keratitis: Isolated microbes and their antibiotic susceptibility pattern during 2004-2014 in region örebro county, sweden. *Acta Ophthalmol*.

SAGLK, A., UCAKHAN, O. O. & KANPOLAT, A. 2013. Ultraviolet a and riboflavin therapy as an adjunct in corneal ulcer refractory to medical treatment. *Eye Contact Lens,* 39**,** 413-5.

SAHAY, P., GOEL, S., NAGPAL, R., MAHARANA, P. K., SINHA, R., AGARWAL, T., et al. 2020. Infectious keratitis caused by rare and emerging micro-organisms. *Curr Eye Res,* 45**,** 761-773.

SAID, D. G., ELALFY, M. S., GATZIOUFAS, Z., EL-ZAKZOUK, E. S., HASSAN, M. A., SAIF, M. Y., et al. 2014. Collagen cross-linking with photoactivated riboflavin (pack-cxl) for the treatment of advanced infectious keratitis with corneal melting. *Ophthalmology,* 121**,** 1377-82.

SAID, D. G., RALLIS, K. I., AL-AQABA, M. A., TING, D. S. J. & DUA, H. S. 2021. Surgical management of infectious keratitis. *Ocul Surf,* doi: 10.1016/j.jtos.2021.09.005.

SAIKIA, K., SRAVANI, Y. D., RAMAKRISHNAN, V. & CHAUDHARY, N. 2017. Highly potent antimicrobial peptides from n-terminal membrane-binding region of e. Coli mreb. *Sci Rep,* 7**,** 42994.

SAMUELSEN, O., HAUKLAND, H. H., ULVATNE, H. & VORLAND, L. H. 2004. Anti-complement effects of lactoferrin-derived peptides. *FEMS Immunol Med Microbiol.,* 41**,** 141-8.

SANTAGATI, M. G., LA TERRA MULE, S., AMICO, C., PISTONE, M., RUSCIANO, D. & ENEA, V. 2005. Lactoferrin expression by bovine ocular surface

epithelia: A primary cell culture model to study lactoferrin gene promoter activity. *Ophthalmic Res.,* 37**,** 270-8.

SAUER, A., GRETH, M., LETSCH, J., BECMEUR, P. H., BORDERIE, V., DAIEN, V., et al. 2020. Contact lenses and infectious keratitis: From a case-control study to a computation of the risk for wearers. *Cornea,* 39**,** 769-774.

SAUER, A., MEYER, N., BOURCIER, T. & KERATITIS, F. S. G. F. C. L. R. M. 2016. Risk factors for contact lens-related microbial keratitis: A case-control multicenter study. *Eye Contact Lens,* 42**,** 158-62.

SAUGAR, J. M., RODRIGUEZ-HERNANDEZ, M. J., DE LA TORRE, B. G., PACHON-IBANEZ, M. E., FERNANDEZ-REYES, M., ANDREU, D., et al. 2006. Activity of cecropin a-melittin hybrid peptides against colistin-resistant clinical strains of acinetobacter baumannii: Molecular basis for the differential mechanisms of action. *Antimicrob Agents Chemother,* 50**,** 1251-6.

SCANZERA, A. C., TU, E. Y. & JOSLIN, C. E. 2020. Acanthamoeba keratitis in minors with orthokeratology (ok) lens use: A case series. *Eye Contact Lens*.

SCHEENSTRA, M. R., VAN HARTEN, R. M., VELDHUIZEN, E. J. A., HAAGSMAN, H. P. & COORENS, M. 2020. Cathelicidins modulate tlr-activation and inflammation. *Front Immunol.,* 11**,** 1137.

SCHNEIDER, P., WALTERS, W. P., PLOWRIGHT, A. T., SIEROKA, N., LISTGARTEN, J., GOODNOW, R. A., JR., et al. 2019. Rethinking drug design in the artificial intelligence era. *Nat Rev Drug Discov*.

SCHNITZLER, E., SPORL, E. & SEILER, T. 2000. Crosslinking of the corneal collagen by uv-radiation with riboflavin for the mode of treatment melting ulcera of the cornea, first results of four patients. [german]. *Klinische Monatsblatter fur Augenheilkunde,* 217**,** 190-193.

SCHOBER, P., BOER, C. & SCHWARTE, L. A. 2018. Correlation coefficients: Appropriate use and interpretation. *Anesth Analg,* 126**,** 1763-1768.

SCHUNEMANN, H., BROZEK, J., GUYATT, G. & OXMAN, A. 2013. *Grade handbook for grading quality of evidence and strength of recommendations.*

SCHUNEMANN, H., BROZEK, J., GUYATT, G., OXMAN, A. & EDITORS. Grade handbook for grading quality of evidence and strength of recommendations. Updated october 2013. The grade working group 2013.

SCOTT, M. G., DAVIDSON, D. J., GOLD, M. R., BOWDISH, D. & HANCOCK, R. E. 2002. The human antimicrobial peptide ll-37 is a multifunctional modulator of innate immune responses. *J Immunol,* 169**,** 3883-91.

SCUDIERO, O., NIGRO, E., CANTISANI, M., COLAVITA, I., LEONE, M., MERCURIO, F. A., et al. 2015. Design and activity of a cyclic mini-beta-defensin analog: A novel antimicrobial tool. *Int J Nanomedicine,* 10**,** 6523-39.

SEAL, D. V., KIRKNESS, C. M., BENNETT, H. G. & PETERSON, M. 1999. Population-based cohort study of microbial keratitis in scotland: Incidence and features. *Cont Lens Anterior Eye,* 22**,** 49-57.

SEMPLE, F. & DORIN, J. R. 2012. Beta-defensins: Multifunctional modulators of infection, inflammation and more? *J Innate Immun.,* 4**,** 337-48.

SEO, J., BYUN, W. Y., ALISAFAEI, F., GEORGESCU, A., YI, Y. S., MASSARO-GIORDANO, M., et al. 2019. Multiscale reverse engineering of the human ocular surface. *Nat Med,* 25**,** 1310-1318.

SHAH, A., SACHDEV, A., COGGON, D. & HOSSAIN, P. 2011. Geographic variations in microbial keratitis: An analysis of the peer-reviewed literature. *Br J Ophthalmol.,* 95**,** 762-7.

SHANKAR, J., SUEKE, H., WIEHLMANN, L., HORSBURGH, M. J., TUFT, S., NEAL, T. J., et al. 2012. 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,* 334**,** 79-86.

SHARMA, S., KUNIMOTO, D. Y., GOPINATHAN, U., ATHMANATHAN, S., GARG, P. & RAO, G. N. 2002. Evaluation of corneal scraping smear examination

methods in the diagnosis of bacterial and fungal keratitis: A survey of eight years of laboratory experience. *Cornea,* 21**,** 643-7.

SHER KHAN, R., IQBAL, A., MALAK, R., SHEHRYAR, K., ATTIA, S., AHMED, T., et al. 2019. Plant defensins: Types, mechanism of action and prospects of genetic engineering for enhanced disease resistance in plants. *3 Biotech,* 9**,** 192.

SHETTY, R., NAGARAJA, H., JAYADEV, C., SHIVANNA, Y. & KUGAR, T. 2014. Collagen crosslinking in the management of advanced non-resolving microbial keratitis. *Br J Ophthalmol,* 98**,** 1033-5.

SHI, W., CHEN, M. & XIE, L. 2007. Amniotic membrane transplantation combined with antiviral and steroid therapy for herpes necrotizing stromal keratitis. *Ophthalmology,* 114**,** 1476-1481.e1.

SHIMA, S., MATSUOKA, H., IWAMOTO, T. & SAKAI, H. 1984. Antimicrobial action of epsilon-poly-l-lysine. *J Antibiot (Tokyo),* 37**,** 1449-55.

SHIMIZU, D., MIYAZAKI, D., EHARA, F., SHIMIZU, Y., UOTANI, R., INATA, K., et al. 2019. Effectiveness of 16s ribosomal DNA real-time pcr and sequencing for diagnosing bacterial keratitis. *Graefes Arch Clin Exp Ophthalmol*.

SHIN, S. Y., KANG, J. H. & HAHM, K. S. 1999. Structure-antibacterial, antitumor and hemolytic activity relationships of cecropin a-magainin 2 and cecropin a-melittin hybrid peptides. *J Pept Res,* 53**,** 82-90.

SHOVAL, O., SHEFTEL, H., SHINAR, G., HART, Y., RAMOTE, O., MAYO, A., et al. 2012. Evolutionary trade-offs, pareto optimality, and the geometry of phenotype space. *Science,* 336**,** 1157-60.

SHUKLA, S. C., SINGH, A., PANDEY, A. K. & MISHRA, A. 2012. Review on production and medical applications of ε-polylysine. *Biochem Eng J,* 65**,** 70-81.

SIBLEY, D. & LARKIN, D. F. P. 2020. Update on herpes simplex keratitis management. *Eye (Lond),* 34**,** 2219-2226.

SIEPRAWSKA-LUPA, M., MYDEL, P., KRAWCZYK, K., WOJCIK, K., PUKLO, M., LUPA, B., et al. 2004. Degradation of human antimicrobial peptide ll-37 by staphylococcus aureus-derived proteinases. *Antimicrob Agents Chemother,* 48**,** 4673-9.

SILHAVY, T. J., KAHNE, D. & WALKER, S. 2010. The bacterial cell envelope. *Cold Spring Harb Perspect Biol,* 2**,** a000414.

SINGH, P. K., TACK, B. F., MCCRAY, P. B., JR. & WELSH, M. J. 2000. Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. *Am J Physiol Lung Cell Mol Physiol.,* 279**,** L799-805.

SINGHAL, N., KUMAR, M., KANAUJIA, P. K. & VIRDI, J. S. 2015. Maldi-tof mass spectrometry: An emerging technology for microbial identification and diagnosis. *Front Microbiol,* 6**,** 791.

SITOULA, R. P., SINGH, S. K., MAHASETH, V., SHARMA, A. & LABH, R. K. 2015. Epidemiology and etiological diagnosis of infective keratitis in eastern region of nepal. *Nepal J Ophthalmol,* 7**,** 10-5.

SKAAT, A., ZADOK, D., GOLDICH, Y., VARSSANO, D., BERGER, Y., EZRA-NIMNI, O., et al. 2014. Riboflavin/uva photochemical therapy for severe infectious keratitis. *Eur J Ophthalmol,* 24**,** 21-28.

SMITH, J. E. 1987. Erythrocyte membrane: Structure, function, and pathophysiology. *Vet Pathol,* 24**,** 471-6.

SOMANI, S. N., RONQUILLO, Y. & MOSHIRFAR, M. 2020. Acanthamoeba keratitis. *Statpearls.* Treasure Island (FL).

SOMERVILLE, T. F., CORLESS, C. E., SUEKE, H., NEAL, T. & KAYE, S. B. 2020. 16s ribosomal rna pcr versus conventional diagnostic culture in the investigation of suspected bacterial keratitis. *Transl Vis Sci Technol,* 9**,** 2.

SONG, A., DESHMUKH, R., LIN, H., ANG, M., MEHTA, J. S., CHODOSH, J., et al. 2021. Post-keratoplasty infectious keratitis: Epidemiology, risk factors, management, and outcomes. *Front Med (Lausanne),* 8**,** 707242.

SONG, X., XIE, L., TAN, X., WANG, Z., YANG, Y., YUAN, Y., et al. 2014. A multi-center, cross-sectional study on the burden of infectious keratitis in china. *PLoS One.,* 9**,** e113843.

SØRENSEN, O. E., GRAM, L., JOHNSEN, A. H., ANDERSSON, E., BANGSBØLL, S., TJABRINGA, G. S., et al. 2003. Processing of seminal plasma hcap-18 to all-38 by gastricsin: A novel mechanism of generating antimicrobial peptides in vagina. *J Biol Chem.,* 278**,** 28540-6.

SORENSEN, O. E., THAPA, D. R., ROSENTHAL, A., LIU, L., ROBERTS, A. A. & GANZ, T. 2005. Differential regulation of beta-defensin expression in human skin by microbial stimuli. *J Immunol.,* 174**,** 4870-9.

SORKHABI, R., SEDGIPOOR, M. & MAHDAVIFARD, A. 2013. Collagen cross-linking for resistant corneal ulcer. *Int Ophthalmol,* 33**,** 61-66.

SPELSBERG, H. & REICHELT, J. A. 2008. Amniotic membrane transplantation in proven ulcerative herpetic keratitis: Successful anti-inflammatory treatment in time. [german]. *Klinische Monatsblatter fur Augenheilkunde,* 225**,** 75-79.

SPOERL, E., MROCHEN, M., SLINEY, D., TROKEL, S. & SEILER, T. 2007. Safety of uva-riboflavin cross-linking of the cornea. *Cornea,* 26**,** 385-9.

SPOHN, R., DARUKA, L., LAZAR, V., MARTINS, A., VIDOVICS, F., GREZAL, G., et al. 2019. Integrated evolutionary analysis reveals antimicrobial peptides with limited resistance. *Nat Commun,* 10**,** 4538.

SRINIVASAN, M., MASCARENHAS, J., RAJARAMAN, R., RAVINDRAN, M., LALITHA, P., GLIDDEN, D. V., et al. 2012. The steroids for corneal ulcers trial: Study design and baseline characteristics. *Arch Ophthalmol,* 130**,** 151-7.

SRIVASTAVA, S. & GILL, A. 2020. Untreated morbidity and treatment-seeking behaviour among the elderly in india: Analysis based on national sample survey 2004 and 2014. *SSM Popul Health,* 10**,** 100557.

STAPLETON, F. 2020. Contact lens-related corneal infection in australia. *Clin Exp Optom,* 103**,** 408-417.

STAPLETON, F., ALVES, M., BUNYA, V. Y., JALBERT, I., LEKHANONT, K., MALET, F., et al. 2017a. Tfos dews ii epidemiology report. *Ocul Surf,* 15**,** 334-365.

STAPLETON, F., EDWARDS, K., KEAY, L., NADUVILATH, T., DART, J. K., BRIAN, G., et al. 2012. Risk factors for moderate and severe microbial keratitis in daily wear contact lens users. *Ophthalmology,* 119**,** 1516-21.

STAPLETON, F., NADUVILATH, T., KEAY, L., RADFORD, C., DART, J., EDWARDS, K., et al. 2017b. Risk factors and causative organisms in microbial keratitis in daily disposable contact lens wear. *PLoS One,* 12**,** e0181343.

STARR, C. G., HE, J. & WIMLEY, W. C. 2016. Host cell interactions are a significant barrier to the clinical utility of peptide antibiotics. *ACS Chem Biol,* 11**,** 3391-3399.

STARR, C. G. & WIMLEY, W. C. 2017. Antimicrobial peptides are degraded by the cytosolic proteases of human erythrocytes. *Biochim Biophys Acta Biomembr,* 1859**,** 2319-2326.

STEINSTRAESSER, L., KOEHLER, T., JACOBSEN, F., DAIGELER, A., GOERTZ, O., LANGER, S., et al. 2008. Host defense peptides in wound healing. *Mol Med.,* 14**,** 528-37.

STERNE, J. A., HERNAN, M. A., REEVES, B. C., SAVOVIC, J., BERKMAN, N. D., VISWANATHAN, M., et al. 2016a. Robins-i: A tool for assessing risk of bias in non-randomised studies of interventions. *Bmj,* 355**,** i4919.

STERNE, J. A., HERNÁN, M. A., REEVES, B. C., SAVOVIĆ, J., BERKMAN, N. D., VISWANATHAN, M., et al. 2016b. Robins-i: A tool for assessing risk of bias in non-randomised studies of interventions. *BMJ.,* 355**,** i4919.

STERNE, J. A. C., SAVOVIĆ, J., PAGE, M. J., ELBERS, R. G., BLENCOWE, N. S., BOUTRON, I., et al. 2019. Rob 2: A revised tool for assessing risk of bias in randomised trials. *BMJ.,* 366**,** l4898.

STOTZ, H. U., THOMSON, J. G. & WANG, Y. 2009. Plant defensins: Defense, development and application. *Plant Signal Behav.,* 4**,** 1010-2.

STRAHL, H. & ERRINGTON, J. 2017. Bacterial membranes: Structure, domains, and function. *Annu Rev Microbiol,* 71**,** 519-538.

SUN, X. G., ZHANG, Y., LI, R., WANG, Z. Q., LUO, S. Y., JIN, X. Y., et al. 2004. Etiological analysis on ocular fungal infection in the period of 1989 - 2000. *Chin Med J (Engl),* 117**,** 598-600.

SUZUKI, T., SUTANI, T., NAKAI, H., SHIRAHIGE, K. & KINOSHITA, S. 2020. The microbiome of the meibum and ocular surface in healthy subjects. *Invest Ophthalmol Vis Sci,* 61**,** 18.

SVENSON, J., BRANDSDAL, B. O., STENSEN, W. & SVENDSEN, J. S. 2007. Albumin binding of short cationic antimicrobial micropeptides and its influence on the in vitro bactericidal effect. *J Med Chem,* 50**,** 3334-9.

TABATABAEI, S. A., SOLEIMANI, M., BEHROUZ, M. J., TORKASHVAND, A., ANVARI, P. & YASERI, M. 2017. A randomized clinical trial to evaluate the usefulness of amniotic membrane transplantation in bacterial keratitis healing. *Ocul Surf,* 15**,** 218-226.

TABIBIAN, D., RICHOZ, O., RIAT, A., SCHRENZEL, J. & HAFEZI, F. 2014. Accelerated photoactivated chromophore for keratitis-corneal collagen cross-linking as a first-line and sole treatment in early fungal keratitis. *Journal of Refractive Surgery,* 30**,** 855-857.

TALL, Y. A., AL-RAWASHDEH, B., ABUALHAIJAA, A., ALMAAYTAH, A., MASADEH, M. & ALZOUBI, K. H. 2020. Functional characterization of a novel hybrid peptide with high potency against gram-negative bacteria. *Curr Pharm Des*.

TAM, A. L. C., CÔTÉ, E., SALDANHA, M., LICHTINGER, A. & SLOMOVIC, A. R. 2017. Bacterial keratitis in toronto: A 16-year review of the microorganisms isolated and the resistance patterns observed. *Cornea,* 36**,** 1528-1534.

TAN, D. T., DART, J. K., HOLLAND, E. J. & KINOSHITA, S. 2012. Corneal transplantation. *Lancet,* 379**,** 1749-61.

TAN, S. Z., WALKDEN, A., AU, L., FULLWOOD, C., HAMILTON, A., QAMRUDDIN, A., et al. 2017. Twelve-year analysis of microbial keratitis trends at a uk tertiary hospital. *Eye (Lond),* 31**,** 1229-1236.

TAVASSOLI, S., NAYAR, G., DARCY, K., GRZEDA, M., LUCK, J., WILLIAMS, O. M., et al. 2019. An 11-year analysis of microbial keratitis in the south west of england using brain-heart infusion broth. *Eye (Lond),* 33**,** 1619-25.

TEMPEST-ROE, S., JOSHI, L., DICK, A. D. & TAYLOR, S. R. 2013. Local therapies for inflammatory eye disease in translation: Past, present and future. *BMC Ophthalmol,* 13**,** 39.

THOMAS, R. K., MELTON, R. & ASBELL, P. A. 2019. Antibiotic resistance among ocular pathogens: Current trends from the armor surveillance study (2009-2016). *Clin Optom (Auckl).* 11**,** 15-26.

THULASI, P., SAEED, H. N., RAPUANO, C. J., HOU, J. H., APPENHEIMER, A. B., CHODOSH, J., et al. 2021. Oral miltefosine as salvage therapy for refractory acanthamoeba keratitis. *Am J Ophthalmol,* 223**,** 75-82.

THURET, G., COURRIER, E., POINARD, S., GAIN, P., BAUD'HUIN, M., MARTINACHE, I., et al. 2020. One threat, different answers: The impact of covid-19 pandemic on cornea donation and donor selection across europe. *Br J Ophthalmol.,* doi: 10.1136/bjophthalmol-2020-317938.

TING, D. S., POTTS, J., JONES, M., LAWTHER, T., ARMITAGE, W. J. & FIGUEIREDO, F. C. 2016a. Changing trend in the utilisation rate of donated corneas for keratoplasty in the uk: The north east england study. *Eye (Lond),* 30**,** 1475-1480.

TING, D. S., POTTS, J., JONES, M., LAWTHER, T., ARMITAGE, W. J. & FIGUEIREDO, F. C. 2016b. Impact of telephone consent and potential for eye donation in the uk: The newcastle eye centre study. *Eye (Lond),* 30**,** 342-8.

TING, D. S., SAU, C. Y., SRINIVASAN, S., RAMAESH, K., MANTRY, S. & ROBERTS, F. 2012. Changing trends in keratoplasty in the west of scotland: A 10-year review. *Br J Ophthalmol.,* 96**,** 405-8.

TING, D. S. J., ANG, M., MEHTA, J. S. & TING, D. S. W. 2019a. Artificial intelligence-assisted telemedicine platform for cataract screening and management: A potential model of care for global eye health. *Br J Ophthalmol,* 103**,** 1537-1538.

TING, D. S. J., BANDYOPADHYAY, J. & PATEL, T. 2019b. Microbial keratitis complicated by acute hydrops following corneal collagen cross-linking for keratoconus. *Clin Exp Optom,* 102**,** 434-436.

TING, D. S. J., BEUERMAN, R. W., DUA, H. S., LAKSHMINARAYANAN, R. & MOHAMMED, I. 2020a. Strategies in translating the therapeutic potentials of host defense peptides. *Front Immunol,* 11**,** 983.

TING, D. S. J., BIGNARDI, G., KOERNER, R., IRION, L. D., JOHNSON, E., MORGAN, S. J., et al. 2019c. Polymicrobial keratitis with cryptococcus curvatus, candida parapsilosis, and stenotrophomonas maltophilia after penetrating keratoplasty: A rare case report with literature review. *Eye Contact Lens,* 45**,** e5-e10.

TING, D. S. J., CAIRNS, J., GOPAL, B. P., HO, C. S., KRSTIC, L., ELSAHN, A., et al. 2021a. Risk factors, clinical outcomes and prognostic factors of bacterial keratitis: The nottingham infectious keratitis study. *Front Med (Lausanne),* 8**,** 715118.

TING, D. S. J., CHEN, Y. & FIGUEIREDO, F. C. 2021b. Effects of whole globe enucleation versus in situ corneoscleral excision on donor cornea tissue quality: A systematic review protocol. *JBI Evid Synth,* 19**,** 251-256.

TING, D. S. J., FOO, V. H., YANG, L. W. Y., SIA, J. T., ANG, M., LIN, H., et al. 2021c. Artificial intelligence for anterior segment diseases: Emerging applications in ophthalmology. *Br J Ophthalmol.,* 105**,** 158-168.

TING, D. S. J., GALAL, M., KULKARNI, B., ELALFY, M. S., LAKE, D., HAMADA, S., et al. 2021d. Clinical characteristics and outcomes of fungal keratitis in the united kingdom 2011-2020: A 10-year study. *J Fungi (Basel),* 7.

TING, D. S. J., GHOSH, N. & GHOSH, S. 2019d. Herpes zoster ophthalmicus. *BMJ,* 364**,** k5234.

TING, D. S. J. & GHOSH, S. 2019. Acute corneal perforation 1 week following uncomplicated cataract surgery: The implication of undiagnosed dry eye disease and topical nsaids. *Ther Adv Ophthalmol.,* 11**,** 2515841419869508.

TING, D. S. J., GOH, E. T. L., MAYANDI, V., BUSOY, J. M. F., AUNG, T. T., PERIAYAH, M. H., et al. 2021e. Hybrid derivative of cathelicidin and human beta defensin-2 against gram-positive bacteria: A novel approach for the treatment of bacterial keratitis. *Sci Rep,* 11**,** 18304.

TING, D. S. J., GOPAL, B. P., DESHMUKH, R., SEITZMAN, G. D., SAID, D. G. & DUA, H. S. 2021f. Diagnostic armamentarium of infectious keratitis: A comprehensive review. *Ocul Surf,* 23**,** 27-39.

TING, D. S. J., HENEIN, C., SAID, D. G. & DUA, H. S. 2019e. Photoactivated chromophore for infectious keratitis-corneal cross-linking (pack-cxl): A systematic review and meta-analysis. *Ocul Surf.,* 17**,** 624-634.

TING, D. S. J., HENEIN, C., SAID, D. G. & DUA, H. S. 2020b. Effectiveness and safety of early adjuvant amniotic membrane transplant versus standard antimicrobial treatment for infectious keratitis: A systematic review protocol. *JBI Evid Synth.,* 18**,** 1808-1814.

TING, D. S. J., HENEIN, C., SAID, D. G. & DUA, H. S. 2020c. Effectiveness of adjuvant photoactivated chromophore corneal collagen cross-linking versus standard antimicrobial treatment for infectious keratitis: A systematic review protocol. *JBI Evid Synth,* 18**,** 194-199.

TING, D. S. J., HENEIN, C., SAID, D. G. & DUA, H. S. 2020d. Re: Prajna et al.: Cross-linking-assisted infection reduction (clair): A randomized clinical trial

evaluating the effect of adjuvant cross-linking on outcomes in fungal keratitis (ophthalmology. 2020;127:159-166). *Ophthalmology,* 127**,** e55-e56.

TING, D. S. J., HENEIN, C., SAID, D. G. & DUA, H. S. 2021g. Amniotic membrane transplantation for infectious keratitis: A systematic review and meta-analysis. *Sci Rep,* 11**,** 13007.

TING, D. S. J., HO, C. S., CAIRNS, J., ELSAHN, A., AL-AQABA, M., BOSWELL, T., et al. 2021h. 12-year analysis of incidence, microbiological profiles and in vitro antimicrobial susceptibility of infectious keratitis: The nottingham infectious keratitis study. *Br J Ophthalmol.,* 105**,** 328-333.

TING, D. S. J., HO, C. S., CAIRNS, J., GOPAL, B. P., ELSAHN, A., AL-AQABA, M., et al. 2021i. Seasonal patterns of incidence, demographic factors and microbiological profiles of infectious keratitis: The nottingham infectious keratitis study. *Eye (Lond),* 35**,** 2543-9.

TING, D. S. J., HO, C. S., DESHMUKH, R., SAID, D. G. & DUA, H. S. 2021j. Infectious keratitis: An update on epidemiology, causative microorganisms, risk factors, and antimicrobial resistance. *Eye (Lond),* 35**,** 1084-1101.

TING, D. S. J., LI, J., VERMA, C. S., GOH, E. T. L., NUBILE, M., MASTROPASQUA, L., et al. 2021k. Evaluation of host defense peptide (cad23)-antibiotic interaction and mechanism of action: Insights from experimental and molecular dynamics simulations studies. *Front Pharmacol,* 12**,** 731499.

TING, D. S. J., LIU, Y. C., LEE, Y. F., JI, A. J. S., TAN, T. E., HTOON, H. M., et al. 2021l. Cosmetic outcome of femtosecond laser-assisted pterygium surgery. *Eye Vis (Lond),* 8**,** 7.

TING, D. S. J., MCKENNA, M., SADIQ, S. N., MARTIN, J., MUDHAR, H. S., MEENEY, A., et al. 2020e. Arthrographis kalrae keratitis complicated by endophthalmitis: A case report with literature review. *Eye Contact Lens,* 46**,** e59-e65.

TING, D. S. J., PEH, G. S. L., ADNAN, K. & MEHTA, J. S. 2021m. Translational and regulatory challenges of corneal endothelial cell therapy: A global perspective. *Tissue Eng Part B Rev,* doi: 10.1089/ten.TEB.2020.0319.

TING, D. S. J., RANA-RAHMAN, R., CHEN, Y., BELL, D., DANJOUX, J. P., MORGAN, S. J., et al. 2019f. Effectiveness and safety of accelerated (9 mw/cm(2)) corneal collagen cross-linking for progressive keratoconus: A 24-month follow-up. *Eye (Lond),* 33**,** 812-818.

TING, D. S. J., RANA-RAHMAN, R., NG, J. Y., WILKINSON, D. J. P., AH-KINE, D. & PATEL, T. 2021n. Clinical spectrum and outcomes of ocular and periocular complications following external-beam radiotherapy for inoperable malignant maxillary sinus tumors. *Ocul Oncol Pathol,* 7**,** 36-43.

TING, D. S. J., SAID, D. G. & DUA, H. S. 2019g. Interface haze after descemet stripping automated endothelial keratoplasty. *JAMA Ophthalmol.,* 137**,** 1201-1202.

TING, D. S. J., SETTLE, C., MORGAN, S. J., BAYLIS, O. & GHOSH, S. 2018. A 10-year analysis of microbiological profiles of microbial keratitis: The north east england study. *Eye (Lond),* 32**,** 1416-1417.

TONG, W., CHEN, D., CHAI, C., TAN, A. M. & MANOTOSH, R. 2019. Disease patterns of microbial keratitis in singapore: A retrospective case series. *Cont Lens Anterior Eye,* 42**,** 455-461.

TRINH, T., MIMOUNI, M., SANTAELLA, G., COHEN, E. & CHAN, C. C. 2021. Surgical management of the ocular surface in neurotrophic keratopathy: Amniotic membrane, conjunctival grafts, lid surgery, and neurotization. *Eye Contact Lens,* 47**,** 149-153.

TRUONG, D. T., BUI, M. T., MEMON, P. & CAVANAGH, H. D. 2015. Microbial keratitis at an urban public hospital: A 10-year update. *J Clin Exp Ophthalmol,* 6.

TSAI, C. W., HSU, N. Y., WANG, C. H., LU, C. Y., CHANG, Y., TSAI, H. H., et al. 2009. Coupling molecular dynamics simulations with experiments for the rational design of indolicidin-analogous antimicrobial peptides. *J Mol Biol,* 392**,** 837-54.

TSAI, P. S., EVANS, J. E., GREEN, K. M., SULLIVAN, R. M., SCHAUMBERG, D. A., RICHARDS, S. M., et al. 2006. Proteomic analysis of human meibomian gland secretions. *Br J Ophthalmol.,* 90**,** 372-7.

TSAI, S. H., LIN, Y. C., HSU, H. C. & CHEN, Y. M. 2016. Subconjunctival injection of fluconazole in the treatment of fungal alternaria keratitis. *Ocul Immunol Inflamm,* 24**,** 103-6.

TSUGITA, A., OKADA, Y. & UEHARA, K. 1965. Photosensitized inactivation of ribonucleic acids in the presence of riboflavin. *Biochim Biophys Acta,* 103**,** 360-3.

TU, E. Y., JOSLIN, C. E., NIJM, L. M., FEDER, R. S., JAIN, S. & SHOFF, M. E. 2009. Polymicrobial keratitis: Acanthamoeba and infectious crystalline keratopathy. *Am J Ophthalmol,* 148**,** 13-9.e2.

TULI, S., GRAY, M. & SHAH, A. 2018. Surgical management of herpetic keratitis. *Curr Opin Ophthalmol,* 29**,** 347-354.

TULI, S. S., SCHULTZ, G. S. & DOWNER, D. M. 2007. Science and strategy for preventing and managing corneal ulceration. *Ocul Surf.,* 5**,** 23-39.

UDDARAJU, M., MASCARENHAS, J., DAS, M. R., RADHAKRISHNAN, N., KEENAN, J. D., PRAJNA, L., et al. 2015. Corneal cross-linking as an adjuvant therapy in the management of recalcitrant deep stromal fungal keratitis: A randomized trial. *Am J Ophthalmol,* 160**,** 131-4.e5.

UETA, M. & KINOSHITA, S. 2010. Innate immunity of the ocular surface. *Brain Res Bull.,* 81**,** 219-28.

UNG, L., ACHARYA, N. R., AGARWAL, T., ALFONSO, E. C., BAGGA, B., BISPO, P. J., et al. 2019a. Infectious corneal ulceration: A proposal for neglected tropical disease status. *Bull World Health Organ,* 97**,** 854-856.

UNG, L., BISPO, P. J. M., DOAN, T., VAN GELDER, R. N., GILMORE, M. S., LIETMAN, T., et al. 2020a. Clinical metagenomics for infectious corneal ulcers: Rags to riches? *Ocul Surf,* 18**,** 1-12.

UNG, L., BISPO, P. J. M., SHANBHAG, S. S., GILMORE, M. S. & CHODOSH, J. 2019b. The persistent dilemma of microbial keratitis: Global burden, diagnosis, and antimicrobial resistance. *Surv Ophthalmol,* 64**,** 255-271.

UNG, L., WANG, Y., VANGEL, M., DAVIES, E. C., GARDINER, M., BISPO, P. J. M., et al. 2020b. Validation of a comprehensive clinical algorithm for the assessment and treatment of microbial keratitis. *Am J Ophthalmol,* 214**,** 97-109.

UPADHYAY, M. P., KARMACHARYA, P. C., KOIRALA, S., SHAH, D. N., SHAKYA, S., SHRESTHA, J. K., et al. 2001. The bhaktapur eye study: Ocular trauma and antibiotic prophylaxis for the prevention of corneal ulceration in nepal. *Br J Ophthalmol,* 85**,** 388-92.

VAJPAYEE, R. B., SHAFI, S. N., MAHARANA, P. K., SHARMA, N. & JHANJI, V. 2015. Evaluation of corneal collagen cross-linking as an additional therapy in mycotic keratitis. *Clin Exp Ophthalmol,* 43**,** 103-107.

VAN HERENDAEL, B. J., OBERTI, C. & BROSENS, I. 1978. Microanatomy of the human amniotic membranes. A light microscopic, transmission, and scanning electron microscopic study. *Am J Obstet Gynecol,* 131**,** 872-80.

VENKATESH, M., BARATHI, V. A., GOH, E. T. L., ANGGARA, R., FAZIL, M., NG, A. J. Y., et al. 2017. Antimicrobial activity and cell selectivity of synthetic and biosynthetic cationic polymers. *Antimicrob Agents Chemother,* 61.

VENTOLA, C. L. 2015. The antibiotic resistance crisis: Part 1: Causes and threats. *P t,* 40**,** 277-83.

VITAL, M. C., BELLOSO, M., PRAGER, T. C. & LANIER, J. D. 2007. Classifying the severity of corneal ulcers by using the "1, 2, 3" rule. *Cornea,* 26**,** 16-20.

VOLA, M. E., MORIYAMA, A. S., LISBOA, R., VOLA, M. M., HIRAI, F. E., BISPO, P. J., et al. 2013. Prevalence and antibiotic susceptibility of methicillin-resistant staphylococcus aureus in ocular infections. *Arq Bras Oftalmol,* 76**,** 350-3.

WADDELL, K. M. 1998. Childhood blindness and low vision in uganda. *Eye (Lond),* 12 (Pt 2)**,** 184-92.

WADE, D., ANDREU, D., MITCHELL, S. A., SILVEIRA, A. M., BOMAN, A., BOMAN, H. G., et al. 1992. Antibacterial peptides designed as analogs or hybrids of cecropins and melittin. *Int J Pept Protein Res,* 40**,** 429-36.

WADE, D., BOMAN, A., WAHLIN, B., DRAIN, C. M., ANDREU, D., BOMAN, H. G., et al. 1990. All-d amino acid-containing channel-forming antibiotic peptides. *Proc Natl Acad Sci U S A,* 87**,** 4761-5.

WADE, H. M., DARLING, L. E. O. & ELMORE, D. E. 2019. Hybrids made from antimicrobial peptides with different mechanisms of action show enhanced membrane permeabilization. *Biochim Biophys Acta Biomembr,* 1861**,** 182980.

WALKDEN, A., FULLWOOD, C., TAN, S. Z., AU, L., ARMSTRONG, M., BRAHMA, A. K., et al. 2018. Association between season, temperature and causative organism in microbial keratitis in the uk. *Cornea,* 37**,** 1555-1560.

WAN, M. J. & HUO, M. 2010. Biological amniotic membrane transplantation combined with remedial contact lens in corneal ulcer. *Int J Ophthalmol.,* 10**,** 931-932.

WAN, X., WANG, W., LIU, J. & TONG, T. 2014. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med Res Methodol,* 14**,** 135.

WANG, B., YANG, S., ZHAI, H. L., ZHANG, Y. Y., CUI, C. X., WANG, J. Y., et al. 2018. A comparative study of risk factors for corneal infection in diabetic and non-diabetic patients. *Int J Ophthalmol,* 11**,** 43-47.

WANG, C., YANG, C., CHEN, Y. C., MA, L. & HUANG, K. 2019a. Rational design of hybrid peptides: A novel drug design approach. *Curr Med Sci,* 39**,** 349-355.

WANG, G. 2008a. Nmr of membrane-associated peptides and proteins. *Curr Protein Pept Sci,* 9**,** 50-69.

WANG, G. 2008b. Structures of human host defense cathelicidin ll-37 and its smallest antimicrobial peptide kr-12 in lipid micelles. *J Biol Chem.,* 283**,** 32637-43.

WANG, G. 2012. Post-translational modifications of natural antimicrobial peptides and strategies for peptide engineering. *Curr Biotechnol,* 1**,** 72-79.

WANG, G. 2014. Human antimicrobial peptides and proteins. *Pharmaceuticals (Basel).* 7**,** 545-94.

WANG, G., ELLIOTT, M., COGEN, A. L., EZELL, E. L., GALLO, R. L. & HANCOCK, R. E. 2012a. Structure, dynamics, and antimicrobial and immune modulatory activities of human ll-23 and its single-residue variants mutated on the basis of homologous primate cathelicidins. *Biochemistry,* 51**,** 653-64.

WANG, G., EPAND, R. F., MISHRA, B., LUSHNIKOVA, T., THOMAS, V. C., BAYLES, K. W., et al. 2012b. Decoding the functional roles of cationic side chains of the major antimicrobial region of human cathelicidin ll-37. *Antimicrob Agents Chemother,* 56**,** 845-56.

WANG, G., LI, X. & WANG, Z. 2016. Apd3: The antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res.,* 44**,** D1087-93.

WANG, G., MISHRA, B., EPAND, R. F. & EPAND, R. M. 2014. High-quality 3d structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin ll-37 and its fragments. *Biochim Biophys Acta.,* 1838**,** 2160-72.

WANG, G., NARAYANA, J. L., MISHRA, B., ZHANG, Y., WANG, F., WANG, C., et al. 2019b. Design of antimicrobial peptides: Progress made with human cathelicidin ll-37. *Adv Exp Med Biol,* 1117**,** 215-240.

WANG, L., HAN, L. & YIN, W. 2015. Study of pathogens of fungal keratitis and the sensitivity of pathogenic fungi to therapeutic agents with the disk diffusion method. *Curr Eye Res,* 40**,** 1095-101.

WEI, X. B., WU, R. J., SI, D. Y., LIAO, X. D., ZHANG, L. L. & ZHANG, R. J. 2016. Novel hybrid peptide cecropin a (1-8)-ll37 (17-30) with potential antibacterial activity. *Int J Mol Sci,* 17.

WEINBERG, A., JIN, G., SIEG, S. & MCCORMICK, T. S. 2012. The yin and yang of human beta-defensins in health and disease. *Front Immunol.,* 3**,** 294.

WESTERHOFF, H. V., JURETIC, D., HENDLER, R. W. & ZASLOFF, M. 1989. Magainins and the disruption of membrane-linked free-energy transduction. *Proc Natl Acad Sci U S A,* 86**,** 6597-601.

WHITCHER, J. P. & SRINIVASAN, M. 1997. Corneal ulceration in the developing world--a silent epidemic. *Br J Ophthalmol,* 81**,** 622-3.

WHITCHER, J. P., SRINIVASAN, M. & UPADHYAY, M. P. 2001. Corneal blindness: A global perspective. *Bull World Health Organ,* 79**,** 214-21.

WILHELMUS, K. R., GEE, L., HAUCK, W. W., KURINIJ, N., DAWSON, C. R., JONES, D. B., et al. 2020. Herpetic eye disease study: A controlled trial of topical corticosteroids for herpes simplex stromal keratitis. *Ophthalmology,* 127**,** S5-s18.

WILLCOX, M. D., CHEN, R., KALAISELVAN, P., YASIR, M., RASUL, R., KUMAR, N., et al. 2020. The development of an antimicrobial contact lens - from the laboratory to the clinic. *Curr Protein Pept Sci,* 21**,** 357-68.

WILLCOX, M. D., HUME, E. B., ALIWARGA, Y., KUMAR, N. & COLE, N. 2008. A novel cationic-peptide coating for the prevention of microbial colonization on contact lenses. *J Appl Microbiol,* 105**,** 1817-25.

WNOROWSKA, U., FIEDORUK, K., PIKTEL, E., PRASAD, S. V., SULIK, M., JANION, M., et al. 2020. Nanoantibiotics containing membrane-active human cathelicidin ll-37 or synthetic ceragenins attached to the surface of magnetic

nanoparticles as novel and innovative therapeutic tools: Current status and potential future applications. *J Nanobiotechnology,* 18**,** 3.

WOLLENSAK, G., SPOERL, E. & SEILER, T. 2003. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol,* 135**,** 620-7.

WU, H. P., LUO, S. R., DONG, N., LIU, Z. S., SHANG, X. M. & YAN, L. 2013a. [corneal collagen crosslinking in the treatment of infectious keratitis]. *Zhonghua Yan Ke Za Zhi,* 49**,** 890-5.

WU, R., WANG, Q., ZHENG, Z., ZHAO, L., SHANG, Y., WEI, X., et al. 2014. Design, characterization and expression of a novel hybrid peptides melittin (1-13)-ll37 (17-30). *Mol Biol Rep,* 41**,** 4163-9.

WU, X., YANG, A. H. & FANG, M. Y. 2013b. Clinical effect observation on fresh amniotic membrane transplantation in the treatment of viral keratitis. *Int Eye Sci.,* 13**,** 563-564.

WU, Z., HOOVER, D. M., YANG, D., BOULEGUE, C., SANTAMARIA, F., OPPENHEIM, J. J., et al. 2003. Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. *Proc Natl Acad Sci U S A.,* 100**,** 8880-5.

XIAO, X., WANG, P., LIN, W. Z., JIA, J. H. & CHOU, K. C. 2013. Iamp-2l: A two-level multi-label classifier for identifying antimicrobial peptides and their functional types. *Anal Biochem,* 436**,** 168-77.

XIE, Q., GAO, M. H. & YU, H. 2014. Clinical observation of cryotherapy and amniotic membrane transplantation in the management of fusarium corneal ulcer. *Int Eye Sci.,* 14**,** 1783-1785.

XU, L., SHAO, C., LI, G., SHAN, A., CHOU, S., WANG, J., et al. 2020. Conversion of broad-spectrum antimicrobial peptides into species-specific antimicrobials capable of precisely targeting pathogenic bacteria. *Sci Rep,* 10**,** 944.

YAGCI, A., PALAMAR, M., HILMIOGLU, S. P. & IRKEC, M. 2016. Cross-linking treatment and corneal transplant in refractory acremonium keratitis: Case report. *Exp Clin Transplant,* 14**,** 580-583.

YAN, H. & HANCOCK, R. E. 2001. Synergistic interactions between mammalian antimicrobial defense peptides. *Antimicrob Agents Chemother,* 45**,** 1558-60.

YANG, D., CHEN, Q., ROSENBERG, H. F., RYBAK, S. M., NEWTON, D. L., WANG, Z. Y., et al. 2004. Human ribonuclease a superfamily members, eosinophil-derived neurotoxin and pancreatic ribonuclease, induce dendritic cell maturation and activation. *J Immunol,* 173**,** 6134-42.

YANG, D., CHERTOV, O., BYKOVSKAIA, S. N., CHEN, Q., BUFFO, M. J., SHOGAN, J., et al. 1999. Beta-defensins: Linking innate and adaptive immunity through dendritic and t cell ccr6. *Science.,* 286**,** 525-8.

YANG, D., ROSENBERG, H. F., CHEN, Q., DYER, K. D., KUROSAKA, K. & OPPENHEIM, J. J. 2003. Eosinophil-derived neurotoxin (edn), an antimicrobial protein with chemotactic activities for dendritic cells. *Blood,* 102**,** 3396-403.

YASIR, M., DUTTA, D. & WILLCOX, M. D. P. 2019a. Comparative mode of action of the antimicrobial peptide melimine and its derivative mel4 against pseudomonas aeruginosa. *Sci Rep.,* 9**,** 7063.

YASIR, M., DUTTA, D. & WILLCOX, M. D. P. 2019b. Mode of action of the antimicrobial peptide mel4 is independent of staphylococcus aureus cell membrane permeability. *PLoS One.,* 14**,** e0215703.

YEH, Y. C., CRERAN, B. & ROTELLO, V. M. 2012. Gold nanoparticles: Preparation, properties, and applications in bionanotechnology. *Nanoscale,* 4**,** 1871-80.

YELCHURI, M. L., MADHAVI, B., GOHIL, N., SAJEEV, H. S., VENKATESH PRAJNA, N. & SRINIVASAN, S. 2017. In vitro evaluation of the drug reservoir function of human amniotic membrane using moxifloxacin as a model drug. *Cornea,* 36**,** 594-599.

YILDIZ, E. H., AIRIANI, S., HAMMERSMITH, K. M., RAPUANO, C. J., LAIBSON, P. R., VIRDI, A. S., et al. 2012. Trends in contact lens-related corneal ulcers at a tertiary referral center. *Cornea,* 31**,** 1097-102.

YILDIZ, E. H., NUROZLER, A. B., OZKAN AKSOY, N., ALTIPARMAK, U. E., ONAT, M., KARAGUZEL, H., et al. 2008. Amniotic membrane transplantation: Indications and results. *Eur J Ophthalmol.,* 18**,** 685-690.

YOUNT, N. Y., WEAVER, D. C., LEE, E. Y., LEE, M. W., WANG, H., CHAN, L. C., et al. 2019. Unifying structural signature of eukaryotic alpha-helical host defense peptides. *Proc Natl Acad Sci U S A,* 116**,** 6944-6953.

YU, M. C., HÖFLING-LIMA, A. L. & FURTADO, G. H. 2016. Microbiological and epidemiological study of infectious keratitis in children and adolescents. *Arq Bras Oftalmol,* 79**,** 289-293.

YU, Y., COOPER, C. L., WANG, G., MORWITZER, M. J., KOTA, K., TRAN, J. P., et al. 2020. Engineered human cathelicidin antimicrobial peptides inhibit ebola virus infection. *iScience,* 23**,** 100999.

ZANETTI, M., GENNARO, R. & ROMEO, D. 1995. Cathelicidins: A novel protein family with a common proregion and a variable c-terminal antimicrobial domain. *FEBS Lett,* 374**,** 1-5.

ZANETTI, M., GENNARO, R., SKERLAVAJ, B., TOMASINSIG, L. & CIRCO, R. 2002. Cathelicidin peptides as candidates for a novel class of antimicrobials. *Curr Pharm Des,* 8**,** 779-93.

ZAREI-GHANAVATI, S. & IRANDOOST, F. 2015. Treatment of refractory keratitis after a boston type i keratoprosthesis with corneal collagen cross-linking. *Cornea,* 34**,** 1161-1163.

ZASLOFF, M. 1987. Magainins, a class of antimicrobial peptides from xenopus skin: Isolation, characterization of two active forms, and partial cdna sequence of a precursor. *Proc Natl Acad Sci U S A,* 84**,** 5449-53.

ZASLOFF, M. 2019. Antimicrobial peptides of multicellular organisms: My perspective. *Adv Exp Med Biol.,* 1117**,** 3-6.

ZASLOFF, M., MARTIN, B. & CHEN, H. C. 1988. Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc Natl Acad Sci U S A,* 85**,** 910-3.

ZBIBA, W. & ABDESSLEM, N. B. 2018. Acanthamoeba keratitis: An emerging disease among microbial keratitis in the cap bon region of tunisia. *Exp Parasitol,* 192**,** 42-45.

ZENG, B., WANG, P., XU, L. J., LI, X. Y., ZHANG, H. & LI, G. G. 2014. Amniotic membrane covering promotes healing of cornea epithelium and improves visual acuity after debridement for fungal keratitis. *Int J Ophthalmol.,* 7**,** 785-9.

ZHANG, M. J., WU, Q. S., RAO, Y. P. & LI, T. 2010. Amniotic membrane transplantation for fungal keratitis. *Int J Ophthalmol.,* 10**,** 1799-1800.

ZHANG, S. D., HE, J. N., NIU, T. T., CHAN, C. Y., REN, C. Y., LIU, S. S., et al. 2017a. Bacteriological profile of ocular surface flora in meibomian gland dysfunction. *Ocul Surf,* 15**,** 242-247.

ZHANG, Y., WANG, Z. Q. & SUN, X. G. 2017b. [etiological analysis and in vitro drug sensitivity of bacterial keratitis in northern china in the period of 2006-2015]. *Zhonghua Yan Ke Za Zhi,* 53**,** 662-667.

ZHAO, X., WU, H., LU, H., LI, G. & HUANG, Q. 2013. Lamp: A database linking antimicrobial peptides. *PLoS One.,* 8**,** e66557.

ZHENG, Y., NIYONSABA, F., USHIO, H., IKEDA, S., NAGAOKA, I., OKUMURA, K., et al. 2008. Microbicidal protein psoriasin is a multifunctional modulator of neutrophil activation. *Immunology,* 124**,** 357-67.

ZHOU, L., LIU, S. P., CHEN, L. Y., LI, J., ONG, L. B., GUO, L., et al. 2011. The structural parameters for antimicrobial activity, human epithelial cell

cytotoxicity and killing mechanism of synthetic monomer and dimer analogues derived from hbd3 c-terminal region. *Amino Acids.,* 40**,** 123-33.

ZHU, L., TITONE, R. & ROBERTSON, D. M. 2019. The impact of hyperglycemia on the corneal epithelium: Molecular mechanisms and insight. *Ocul Surf,* 17**,** 644-654.

ZLOTO, O., BAREQUET, I. S., WEISSMAN, A., NIMNI, O. E., BERGER, Y. & AVNI-ZAUBERMAN, N. 2018. Does pack-cxl change the prognosis of resistant infectious keratitis? *J Refract Surg,* 34**,** 559-563.

ZORZI, A., DEYLE, K. & HEINIS, C. 2017. Cyclic peptide therapeutics: Past, present and future. *Curr Opin Chem Biol,* 38**,** 24-29.

APPENDIX

AWARDS AND GRANTS OBTAINED DURING THE PHD

1. **Medical Research Council / Fight for Sight Clinical Research PhD Fellowship** (Role: Principal Investigator; Grant: £295,000 [with grant extension]; Period: 2019-21) *The first recipient in the Section of Academic Ophthalmology, University of Nottingham, UK.
2. **Fight for Sight / John Lee, Royal College of Ophthalmologists (RCOphth) Primer Fellowship** (Role: Principal Investigator; Grant: £60,000; Period: 2018-19) *The first recipient in the UK.
3. **Fight for Sight / Acanthamoeba Keratitis Small Grant Award** (Role: Principal Investigator; Grant: £15,000; Period: 2021-22)
4. **Association for Research in Vision and Ophthalmology (ARVO) Science Communication Training Fellowship** (2020-21) *One of the 10 international candidates selected for this Fellowship.
5. **University of Nottingham (UoN) Vice Chancellor's Medal 2021** (Awarded on the basis of research excellence)
6. **UoN Sue Watson PhD Presentation Award (Student Choice Prize) 2021**
7. **UoN Research Academy Research Travel Prize 2021**
8. **United Kingdom & Ireland Society of Cataract & Refractive Surgeons (UKISCRS) Best Poster Presentation 2021**
9. **University of Nottingham International Research Collaboration Award** (Role: Principal Investigator; Grant: £5000; Period: 2018-19)
10. **Asia Cornea Society Santen Education Grant** (Role: Principal Investigator; Grant: £2500; Period: 2020)

11. **Nottingham Eye Symposium David Meyer Research Prize 2020** (For the best basic science research paper and oral presentation)
12. UK Finalist for the Thea International Contest of Corneal Clinical Cases in Pathologies of the Eye (TROPHY) Award 2020

LIST OF PUBLICATIONS ARISING FROM THE PHD WORK

1. **Ting DSJ**, Ho CS, Deshmukh R, Said DG, Dua HS. Infectious keratitis: An update on epidemiology, causative microorganisms, risk factors, and antimicrobial resistance. *Eye (Lond).* 2021;35(4):1084-1101. (**Chapter 1**)
2. **Ting DSJ**, Beuerman RW, Dua HS, Lakshminarayanan R, Mohammed I. Strategies in translating the therapeutic potentials of host defense peptides. *Front Immunol.* 2020;11:983. (**Chapter 1**)
3. **Ting DSJ**, Mohammed I, Lakshminarayanan R, Beuerman RW, Dua HS. Host defense peptides at the ocular surface: The roles in health and diseases, and therapeutic potentials. *Front Med.* 2021; (Submission) (**Chapter 1**)
4. **Ting DSJ**, Gopal BP, Deshmukh R, Said DG, Dua HS. Diagnostic armamentarium of infectious keratitis: A comprehensive review. *Ocul Surf.* 2021;23:27-39. (**Chapter 1**)
5. **Ting DSJ**, Ho CS, Cairns J, Elsahn A, Al-Aqaba MA, Boswell T, Said DG, Dua HS. 12-year analysis of incidence, microbiological profiles, and *in vitro* antimicrobial susceptibility of infectious keratitis: The Nottingham Infectious Keratitis Study. *Br J Ophthalmol.* 2021;105(3):328-333. (**Chapter 2**)
6. **Ting DSJ**, Ho CS, Cairns J, Gopal BP, Elsahn A, Al-Aqaba MA, Boswell T, Said DG, Dua HS. Seasonal patterns of incidence, demographic factors, and microbiological profiles of infectious keratitis: The Nottingham Infectious Keratitis Study. *Eye (Lond).* 2021;35:2543-2549. (**Chapter 3**)
7. **Ting DSJ**, Cairn J, Ho CS, Gopal BP, Krstic L, Elsahn A, Lister M, Said DG, Dua HS. Risk factors, clinical outcomes and prognostic factors of bacterial keratitis: The Nottingham Infectious Keratitis Study. *Front Med.* 2021;8:715118. (**Chapter 4**)

8. **Ting DSJ**, Henein C, Said DG, Dua HS. Photoactivated chromophore for infectious keratitis-corneal collagen cross-linking (PACK-CXL): A systematic review and meta-analysis. *Ocul Surf.* 2019;17(4):624-34. (**Chapter 5**)
9. **Ting DSJ**, Henein C, Said DG, Dua HS. Effectiveness of adjuvant photoactivated chromophore for infectious keratitis-corneal collagen cross-linking (PACK-CXL) versus standard antimicrobial treatment for infectious keratitis: A systematic review protocol. *JBI Evid Synth.* 2020;18(1):194-9. (**Chapter 5**)
10. **Ting DSJ**, Henein C, Said DG, Dua HS. Re: Prajna et al.: Cross-linking-assisted infection reduction: A randomized clinical trial evaluating the effect of adjuvant cross-linking on outcomes in fungal keratitis (Ophthalmology 2020;127:159-166). *Ophthalmology.* 2020;127(8):e55-e56. (**Chapter 5**)
11. **Ting DSJ**, Henein C, Said DG, Dua HS. Amniotic membrane transplant for infectious keratitis: A systematic review and meta-analysis. *Sci Rep.* 2021;11:13007. (**Chapter 6**)
12. **Ting DSJ**, Henein C, Said DG, Dua HS. Effectiveness and safety of early adjuvant amniotic membrane transplant versus standard antimicrobial therapy for infectious keratitis: A systematic review protocol. *JBI Evid Synth.* 2020;18(8):1808-1814. (**Chapter 6**)
13. **Ting DSJ**, Goh ETL, Mayandi V, Busoy JMF, Aung TT, Periayah MH, Nubile M, Mastropasqua L, Said DG, Htoon HM, Barathi VA, Beuerman RW, Lakshminarayanan R, Mohammed I, Dua HS. Hybrid derivative of cathelicidin and human beta defensin-2 against Gram-positive bacteria: A novel approach for the treatment of bacterial keratitis**.** *Sci Rep.* 2021;11:18304. (**Chapter 7**)
14. **Ting DSJ**, Li J, Verma C, Said DG, Beuerman RW, Lakshminarayanan R, Mohammed I, Dua HS. Evaluation of host defense peptide (CaD23)-antibiotic interaction and mechanism of action: Insights from experimental and molecular dynamics simulations studies. *Front Pharmacol.* 2021;12:731499. (**Chapter 8**)

LIST OF PRESENTATIONS RELATED TO THE PHD

1. **Ting DSJ**, Fakae L, Mohammed I, Dua HS. Human-derived hybrid cathelicidin and human beta-defensin: A potential novel treatment for bacterial and Acanthamoeba keratitis. Poster presentation at the Oxford Ophthalmological Congress; 6 July 2021.
2. **Ting DSJ**, Henein C, Said DG, Dua HS. Photoactivated chromophore for infectious keratitis-corneal collagen cross-linking (PACK-CXL): a systematic review and meta-analysis. Oral presentation at the Oxford Ophthalmological Congress; 6 July 2021.
3. **Ting DSJ**, Said DG, Beuerman RW, Lakshminarayanan R, Mohammed I, Dua HS. Development of hybridised cathelicidin and human beta-defensin as novel treatment for Gram-positive bacterial keratitis. Oral presentation at the University of Nottingham Sue Watson presentation event; 28 May 2021. [**Awarded the Sue Watson Presentation Award (Students' Choice Prize)]**
4. **Ting DSJ**, Said DG, Nubile M, Mastropasqua L, Barathi VA, Beuerman RW, Lakshminarayanan R, Mohammed I, Dua HS. Hybrid derivative of cathelicidin (LL-37) and human beta-defensin against Gram-positive bacteria: A novel approach for the treatment of bacterial keratitis. ARVO 2021; 1-7th May 2021.
5. **Ting DSJ**, Ho CS, Cairns J, Gopal B, Elsahn A, Said DG, Dua HS. Seasonal patterns of incidence, demographic factors, and microbiological profiles of infectious keratitis: the Nottingham Infectious Keratitis Study. Poster presentation at 25th ESCRS Virtual Winter Meeting; 19-21 February 2021.
6. **Ting DSJ**, Lakshminarayanan R, Dua HS, Mohammed I. Potential of human-derived hybrid host defense peptide against Gram-positive bacteria: A novel approach for the treatment of bacterial keratitis. E-presentation at Oxford Ophthalmological Congress, Oxford, UK; 6th July 2020.

7. **Ting DSJ**, Downward L, Curnow E, Dua HS. Five-year graft survival of therapeutic keratoplasty: A 15-year UK corneal transplant registry study. E-presentation at Oxford Ophthalmological Congress, Oxford, UK; 6th July 2020.
8. **Ting DSJ**, Cairns J, Ho CS, Elsahn A, Al-Aqaba MA, Boswell T, Said DG, Dua HS. A 12-year analysis of incidence, microbiological profiles, and *in vitro* antimicrobial susceptibility of infectious keratitis: the Nottingham Eye Study. Poster presentation at ARVO, Baltimore, 3-7 May 2020.
9. **Ting DSJ**, Said DG, Nubile M, Mastropasqua L, Lakshminarayanan R, Beuerman RW, Dua HS, Mohammed I. Potential of Human-Derived Hybrid Host Defense Peptide Against Gram-Positive Bacteria: A Novel Approach for the Treatment of Bacterial Keratitis. Oral presentation at Nottingham Eye Research Symposium; 25th January 2020. **(Awarded the David Meyer Research Prize for best basic science research paper)**
10. **Ting DSJ**, Mohammed I, Dua HS. Human-derived hybrid antimicrobial peptides for corneal infection: Exploring the full potential of human innate immunity. Oral presentation at Nottingham Eye Research Symposium; 25th January 2019.

Appendix 1.

Search strategy for PACK-CXL in MEDLINE

1. Cross-link*.mp
2. Crosslink*.mp
3. CXL.mp
4. KXL.mp
5. Cross-Linking Reagents/
6. Riboflavin*.mp
7. Vitamin B.mp
8. Photosensiti*.mp
9. Keratitis.mp
10. Corneal infect*.mp
11. Corneal ulcer*.mp
12. Exp Keratitis/
13. Exp Corneal Ulcer/
14. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
15. 9 or 10 or 11 or 12 or 13
16. 14 and 15
17. Limit 16 to humans
18. Limit 17 to yr=”2003 – 2019’

Search strategy for PACK-CXL in EMBASE

1. Cross-link*.mp
2. Crosslink*.mp
3. CXL.mp
4. KXL.mp
5. Riboflavin*.mp
6. Vitamin B.mp
7. Photosensiti*.mp
8. Exp cross linking/
9. Keratitis.mp
10. Corneal infect*.mp
11. Corneal ulcer*.mp
12. Exp keratitis/
13. Exp corneal ulcer/
14. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
15. 9 or 10 or 11 or 12 or 13
16. 14 and 15
17. Limit 16 to humans
18. Limit 17 to yr=”2003 – 2019”

Appendix 2. Summary of all randomized control trials evaluating the effectiveness and safety of photoactivated chromophore for infectious keratitis-corneal cross-linking (PACK-CXL).

Authors	Year	Protocol registration*	Age, years	Male gender, %	Total eyes (PACK-CXL)	Total eyes (control)	Causative organisms (in CXL group)					
							B	F	A	V	M	CN
Bamdad et al.	2015	N	Mean = 39.6 (CXL) vs. 40.3 (control)	21 (66%)	16	16	16					
Kasetsuwan et al.	2016	Y	Mean = 45 (CXL) vs. 54 (control)	21 (70%)	15	15	7	8				
Said et al.	2014	N	Mean = 37 (CXL) vs. 50 (control)	18 (45%)	21	19	7	3	1	0	7	3
Uddaraju et al.	2015	Y	Median = 40 (CXL) vs. 56 (control)	8 (61%)	6	7		6				

Authors	Pre-CXL vision (LogMAR)	Time from first presentation to PACK-CXL	CXL treatment protocol	Combined with SAT (for CXL group)?	Severity of ulcer[$]	Follow-up	COI
Bamdad et al.	-	Same day	3mW/cm^2 for 30mins	Y	Mean = 17mm^2 (CXL) vs. 20mm^2 (control)	1 month	N
Kasetsuwan et al.	Mean = 1.8 (CXL) vs. 1.7 (control)	Same day	3mW/cm^2 for 30mins	Y	Median = 31mm^2 (CXL) vs. 31mm^2 (control)	1 month	N
Said et al.	Mean = 2.2 (CXL) vs. 2.0 (control)	Within 48 hours	3mW/cm^2 for 30mins	Y	Mean = 30mm^2 (CXL) vs. 16mm^2)	2 weeks – 4 months	Y
Uddaraju et al.	Median = HM (CXL) vs. HM (control)	After 2 weeks	3mW/cm^2 for 30mins	Y	Deep stromal; median = 7mm (CXL) vs. 5mm (control)	-	N

*Prospective registration of the clinical trial protocol in a publicly accessible database.

B = Bacteria; F = Fungi; A = Acanthamoeba; V = Viruses; M = Mixed infection; CN = Culture-negative presumed infectious keratitis; SAT = Standard antimicrobial treatment; COI = Conflict of interest; IQR = Interquartile range; HM = Hand movement

Appendix 3.

Search strategy for amniotic membrane transplant for infectious keratitis.

Search strategy for MEDLINE

19. Keratitis.mp
20. Corneal infect*.mp
21. Corneal ulcer*.mp
22. Exp Keratitis/
23. Exp Corneal Ulcer/
24. 1 or 2 or 3 or 4 or 5
25. Exp Amnion/
26. Amnion.mp.
27. Amniotic membrane.mp.
28. 7 or 8 or 9
29. 6 and 10
30. Limit 11 to humans

Search strategy for EMBASE

19. Keratitis.mp
20. Corneal infection.mp
21. Corneal infections.mp
22. Corneal ulcer*.mp
23. Exp keratitis/
24. Exp bacterial eye infection
25. Exp amnion/
26. Amniotic membrane.mp
27. 1 or 2 or 3 or 4 or 5 or 6
28. 7 or 8
29. 9 and 10
30. Limit 11 to humans