Understanding and reducing microbial contamination of contact lens cases

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Understanding and Reducing Microbial Contamination of Contact Lens Cases

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M.Phil Optometry

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Abstract 350 words maximum

Microbes often contaminate contact lens storage cases during use. Up to 85% of contact lens storage cases show contamination by bacteria and contaminated lens cases may be associated with corneal infections and inflammation. This thesis sets out to understand how bacteria colonise cases, the kinetics and patterns of bacterial interactions during colonisation and determines how antimicrobial strategies might limit storage case contamination and deliver safer contact lens wear, both in laboratory studies and clinical trials.

The rate of contact lens case contamination was estimated using a relatively new povidone iodine (cleadew™) based disinfecting system. Contact lens case contamination was low, with 30% of cases having no culturable microbes, but higher level of Gram-negative bacteria than some other disinfecting solutions. However, use of cleadew™ resulted in the recovery of multiple bacterial species from the same lens case.

Subsequent experiments were designed to understand how multiple species may attach and colonise lens cases. My research demonstrated for the first time that ocular isolates of *P. aeruginosa*, *S. aureus*, *A. radioresistens*, *M. luteus* and *Staphylococcus* spp. could coaggregate. For *P. aeruginosa* and *S. aureus* coaggregation was not related to the build-up of biofilms in contact lens cases as there was no evidence that the coaggregation was associated with cohesion between the strains. However, *A. radioresistens*, *M. luteus* and *Staphylococcus* spp. coaggregated and cohered. Overall, these findings suggest that coaggregation and cohesion may occur and contribute to the coexistence of different microbial types in contact lens cases.

Then I examined, whether antimicrobial silver-impregnated barrel cases could reduce the level of case contamination *in vitro* and *in vivo*. Silver was very effective in cases and resulted in significant decreases level of microbial colonization and lower levels of multispecies recovery from lens cases.

This thesis revealed that the combined efficacy of silver in cases along with a disinfecting solution could be an approach to reduce overall bacterial contamination, particularly contamination by Gram positive bacteria. Further investigations are required to understand whether the microbes formed multispecies biofilms in lenses cases, and whether the reductions in lens case contamination result in reduced complications during lens wear.

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Dedication

আমার ভালো, মা

To my,

Mom Mrs Anita Datta

and

Dad Mr Satya Ranjan Datta

Abstract

Microbes often contaminate contact lens storage cases during use. Up to 85% of contact lens storage cases show contamination by bacteria and contaminated lens cases may be associated with corneal infections and inflammation. This thesis sets out to understand how bacteria colonise cases, the kinetics and patterns of bacterial interactions during colonisation and determines how antimicrobial strategies might limit storage case contamination and deliver safer contact lens wear, both in laboratory studies and clinical trials.

The rate of contact lens case contamination was estimated using a relatively new povidone iodine (cleadew™) based disinfecting system. Contact lens case contamination was low, with 30% of cases having no culturable microbes. Comparison with previously published data showed that use of cleadew™ resulted in low frequencies of Gram positive (49%) and fungal (8%) contamination and a low, but higher than some other disinfecting solutions, level of Gram-negative bacteria. However, use of cleadew™ resulted in the recovery of multiple bacterial species from the same lens case.

Subsequent experiments were designed to understand how multiple species may attach and colonise lens cases. My research demonstrated for the first time that ocular isolates of *P. aeruginosa*, *S. aureus*, *A. radioresistens*, *M. luteus* and *Staphylococcus* spp. could coaggregate. For *P. aeruginosa* and *S. aureus* coaggregation was not related to the build-up of biofilms in contact lens cases as there was no evidence that the coaggregation was associated with cohesion between the strains. However, *A.*

radioresistens, M. luteus and *Staphylococcus* spp. coaggregated and cohered. Overall, these findings suggest that coaggregation and cohesion may occur and contribute to the coexistence of different microbial types in contact lens cases.

Then I examined, whether antimicrobial silver-impregnated barrel cases could reduce the level of case contamination *in vitro* and *in vivo*. Silver was very effective in cases and resulted in significant decreases level of microbial colonization and lower levels of multispecies recovery from lens cases.

This thesis revealed that the combined efficacy of silver in cases along with a disinfecting solution could be an approach to reduce overall bacterial contamination, particularly contamination by Gram positive bacteria. This reduction in microbial colonisation of cases may reduce certain contact lens induced ocular complications. Further investigations are required to understand whether the microbes formed multispecies biofilms in lenses cases, and whether the reductions in lens case contamination result in reduced complications during lens wear.

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'I Ananya Datta hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

Signed …… ……

Ananya Datta

Date ……

Contents of Thesis

Contents

List of Tables

List of Figures

Glossary of Abbreviations and Terminology

Ag	Silver
AHLs	acyl-homoserine lactone
ATP	Adenosine triphosphate
AMO	Advanced Medical Optics
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
ANZCTR	Australian New Zealand Clinical Trials Registry
AI	autoinducer
Baseline	before lens wear
CDC	Centre for Disease Control
CA	Cetrimide Agar
CBA	Chocolate Blood Agar
CTN	Clinical Trial Notification
CFU	colony forming unit
CI	confidence interval
CL	Contact Lens
CLARE	Contact lens induced acute red eye
CLPU	Contact lens induced peripheral ulcer
CCLRU	Cornea and Contact Lens Research Unit
DW	daily wear
^{o}C	degrees of centigrade
DNA	Deoxyribonucleic acid

DE	Dey Engley Neutralizing Broth
Na_2HPO_4	disodium phosphate
DPBS	Dulbecco's phosphate buffered saline
e.g.	example
EW	Extended wear
EPS	Extracellular polymeric substances
FISH	Fluorescent *in situ* Hybridization
FDA	Food and Drug Administration
H_2O_2	hydrogen peroxide
HEMA	hydroxyethyl methacrylate
HPMC	hydroxypropyl methylcellulose
IK	Infiltrative keratitis
ISO	International Organization for Standardization
LB	Luria Bertani
MSA	Mannitol Salt Agar
w/v	mass per volume
MRSA	methicillin-resistant Staphylococcus aureus
µg	microgram(s)
µl	microliter(s)
µM	micromolar
mg	milligram(s)
min	minutes
KH_2PO_4	Monopotassium phosphate
MPDS	Multipurpose disinfecting solution

ALDOX	myristamidopropyl dimethylamine
nm	Nano-meter(s)
NCTC	National Counter Terrorism Centre
N/A	not applicable
NR	not reported
NA	nutrient agar
OD	Optical density
O_2	oxygen
%	Percentage(s)
PBS	phosphate buffer solution
PHMB	polyaminopropyl biguanide
PCR	Polymerase chain reaction
PQ-1/Polyquad	polyquaternium-1
PIA	Polysaccharide Intercellular Adhesion
KCl	potassium chloride
pH	potential hydrogen
PVP-I	povidone-iodine
QS	Quorum sensing
rpm	rate per minute
RNA	Ribonucleic acid
RGP	Rigid Gas Permeable
SCL	Soft Contact Lens
SDA	Sabouraud's Dextrose Agar
s	second(s)

SiHy	silicone hydrogel
NaCl	sodium chloride
spp.	Species
sp.	Species (single)
SD	standard deviation
SPSS	Statistical Package for Social Science
i.e.	that is
TGA	Therapeutic Goods Administration
TSB	Tripicase Soy Broth
TSA	Trypticase Soy Agar
UV	ultraviolet
UNSW	University of New South Wales
WHO	World Health Organisation

Chapter 1:
Introduction and Literature Review

Chapter 1
Introduction and Literature Review

1.1 Contact Lenses

Since the early 19th century, contact lenses (CL) have been used as a popular biomedical device as an alternative to glasses either to correct refractive error or as a therapeutic or cosmetic device. Different modalities of wear for soft contact lenses are available in the market such as daily wear, extended wear and continuous wear lenses of either hydrogel or silicone hydrogel (SiHy) materials.

Globally 140 million people wear contact lenses (Nichols, 2015) and there are 40.9 million contact lens wearers in the United States with 87% wearing soft lenses and 15% rigid lenses (Cope *et al*., 2015). In the USA in 2015 silicone hydrogels represented 67% of the soft contact lens market compared with 33% for hydrogels. In developed countries, up to 90% of soft lens wearers use contact lenses on a daily basis, with a majority of them using daily disposable (single use lenses, worn during the day and then disposed of) (Efron *et al*., 2010) or biweekly or monthly disposable lenses (Morgan *et al*., 2015). In developing countries such as Iran, Hungary, India and Nepal more than 90% of the population use frequent replacement (three to six monthly) or annual replacement lenses and 8% bi-weekly (Morgan *et al*., 2015) and the remaining wearers either use daily disposables, extended wear lenses or daily use of rigid contact lenses (Nichols, 2015).

1.2 CONTACT LENS ACCESSORIES AND CARE REGIMEN

To maintain safe and successful contact lens wear, contact lenses and other contact lens related accessories including the lens storage cases need to be cleaned and disinfected daily using a contact lens disinfecting system. Multipurpose disinfecting solutions (MPDS) are the most commonly used products with 80% - 90% of wearers using MPDS for daily maintenance of re-usable soft contact lenses (Efron & Morgan, 2008; González-Méijome *et al*., 2007). Another frequently used disinfecting system is one-step or two-step hydrogen peroxide (H_2O_2), which is used as an alternative by patients suffering from allergic or toxic reactions such as redness or irritation to the eye from the ingredients of MPDS (Stuart, 2012). The disinfecting systems used for rigid lenses are different from the care system used for soft lenses (Tellakula, 2009).

Excluding daily disposable and extended wear contact lenses, all other types of lenses need to be stored in lens cases for overnight storage, disinfection and lens hydration. Depending on the disinfecting systems, different designs and sizes of contact lens cases have been manufactured.

1.3 CONTACT LENS CASE CONTAMINATION

Contact lens case contamination has been identified as one of the primary sources of contact lens contamination in both soft and rigid lens wearers (Qu *et al*., 2011; Vermeltfoort *et al*., 2008). The lens case may act as reservoir to transfer the microbes to lenses and then to the eye (Qu *et al*., 2011; Vermeltfoort *et al*., 2008). Contact lens storage cases are frequently contaminated at about 50% (Devonshire *et al*., 1993; Kuzman *et al*., 2014; Wilson *et al*., 1990; Wu *et al*., 2010; Yung *et al*., 2007). The microorganisms causing microbial keratitis have been traced to the lens cases of affected individuals (Mayo *et al*., 1987; McLaughlin-Borlace *et al*., 1998; Stapleton *et*

al., 1995). Lens cases of rigid lens wearers may be more frequently contaminated compared to soft lens wearers (Devonshire *et al*., 1993), despite a low risk of disease (Dart *et al*., 1991).

1.3.1 Commonly isolated Microorganism from Lens Cases:

Multiple genera and species have been isolated from lens cases including bacteria, fungi and protozoa (Boost *et al*., 2005; Dantam *et al*., 2016; Devonshire *et al*., 1993; Fleiszig *et al*., 1996; Gray *et al*., 1995; Jiang *et al*., 2014; Larkin *et al*., 1990; Pens *et al*., 2008; Willcox *et al*., 2010; Yung *et al*., 2007), and bacteria predominate (Dantam *et al*., 2016; Devonshire *et al*., 1993; Gray *et al*., 1995; Jiang *et al*., 2014; Pens *et al*., 2008). The profile and the frequency of isolated microorganisms from lens cases are shown in **Table 1.1**. Most studies have found that contact lens cases of asymptomatic wearers are contaminated with Gram positive bacteria, particularly *Staphylococcus* spp (Fleiszig *et al*., 1996; Gopinathan *et al*., 1997; Hart *et al*., 1987; Willcox *et al*., 2010; Wu *et al*., 2010). *S. aureus* and *Micrococcus* spp. are also commonly isolated from lens cases of asymptomatic wearers (Velasco & Bermudez, 1996; Willcox *et al*., 2010; Wu *et al*., 2010; Yung *et al*., 2007) as well as those cases collected from people with microbial keratitis (Mayo *et al*., 1987; McLaughlin-Borlace *et al*., 1998). Contact lens cases can also be contaminated by Gram negative bacteria such as *P. aeruginosa* and *Serratia* spp. which are more often isolated from the cases of wearers with contact lens induced corneal infiltrates (McLaughlin-Borlace *et al*., 1998; Stapleton *et al*., 1995) and rarely in asymptomatic lens wearers (Thakur & Gaikwad, 2014).

Microbial contamination of contact lens cases has been implicated in both sterile (inflammation without microbial contamination) and microbial keratitis (Bates *et al*., 1989; Bharathi *et al*., 2007; McLaughlin-Borlace *et al*., 1998; Stapleton *et al*.,

1995). However, identification of microorganisms using traditional microbial culture techniques may be incomplete as some microbes are not cultivable (Wiley *et al*., 2012). Using 16s ribosomal RNA (rRNA) gene analysis, can be an alternative advanced technique to identify the contaminants of contact lenses and contact lens case (Wiley *et al*., 2012).

The frequency of isolation of fungi and protozoa from lens cases has been reported to be 24% and 20%, respectively (Gray *et al*., 1995). Among free living amoeba, *Acanthamoeba* have been the most commonly isolated from lens cases, at a frequency of 8% (Devonshire *et al*., 1993; Donzis *et al*., 1987; Gray *et al*., 1995; Larkin *et al*., 1990), and these often coexist with bacteria (Dini *et al*., 2000; Hong *et al*., 2014).

Table 1.1: Profile and frequency of contact lens case recovered microorganisms.

Author	Sample size and type	Type of used disinfecting solution	Profile and Frequency of recovered microorganisms			
			Bacteria		Fungi	Protozoa
			Gram positive bacteria	Gram negative bacteria		
(Callender *et al*., 1986)	58 asymptomatic lens wearers	NR	*Staphylococcus epidermidis* (50%)	*Moraxella* spp. (10%) *Enterobacter* (7%)	NR	NR
(Wilson *et al*., 1990)	118 asymptomatic lens wearers	Polyquaternium-1, Polyquaternium-1, Chlorhexidine, Hydrogen peroxide – based disinfecting systems	*Staphylococcus epidermidis* *Micrococcus* spp.	*Serratia marcescens* *Pseudomonas aeruginosa*	NR	NR
(Fleiszig & Efron, 1992)	84 asymptomatic contact lens wearers (Microbial isolation was done from 72 lens cases)	NR	Not isolated	*Pseudomonas* spp. (14%) *Klebsiella* spp. (11%) *Enterobacter cloacae*	Candida albicans (1 case)	NR
(Devonshire *et al*., 1993)	178 asymptomatic lens wearers	Heat, Chemical, Chlorine, Chlorhexidine, Hydrogen peroxide – based disinfecting systems	Not isolated	*Klebsiella pneumoniae* (11%) *Serratia marcescens* (5%) *Pseudomonas acidovorans* (5%) *Pseudomonas aeruginosa* (2%)	Yeast (1%)	*Acantham oeba* spp. (4.5%)
(Gray *et al*., 1995)	101 asymptomatic wearers	Hydrogen peroxide ('one step'; Aosept) , Hydrogen peroxide ('two step'; Oxysept 1 and 2) Thiomersal (Soaclens, Hydrocare and Boil-n-soak), Polyaminopropyl biguanide	Diphtheroid spp. (11.5%) *Micrococcus* spp. (1.3%) *Bacillus* spp. (1.3%)	*Pseudomonas* spp. (60%) *Serratia* spp. (25%) *Alcaligenes* spp. (26%) *Acinetobacter* spp. (14%)	Fungi (24%)	Protozoan all spp. (24%) *Acantham oeba* spp. (7%)

		0.00005% (Bausch & Lomb Multi-purpose solution), Polyquad (OPTI-FREE), Chlorhexidine (Boston Lens), phenylmercuric nitrate (Clean-n-soak), Anionic amphoteric surfactants (Duraclean) - based disinfecting systems				
(Velasco & Bermudez, 1996)	126 asymptomatic wearers	NR	*Staphylococcus epidermidis* (29%) *Staphylococcus aureus* (17%) *Streptococcus* spp. (13%)	*Pseudomonas aeruginosa* (8%)	NR	NR
(McLaughlin-Borlace *et al*., 1998)	20 microbial keratitis patients (Bacterial isolation in 12 lens cases)	Chlorine, Hydrogen peroxide, Thiomersol, PHMB – based disinfecting systems	Coagulase-negative staphylococci (8%)	*Pseudomonas aeruginosa* (25%) *Serratia marcescens* (8%)	NR	*Acantham oeba* spp.
(Yung *et al*., 2007)	101 asymptomatic lens wearers	Hydrogen peroxide, Chemical – based disinfecting systems	Coagulase-negative staphylococci *S. aureus* *Flavobacterium* spp. *Escherichia coli*	*Serratia marcescens* *Pseudomonas aeruginosa* *Acinetobacter* spp. *Xanthomonas maltophilia*	NR	NR
(Willcox *et al*., 2010)	232 asymptomatic wearers	Polyhexanide 0.0001% (AQuify), Hydrogen peroxide (CLEAR CARE), Polyquaternium-1 0.001% with myristamindopropyl dimethylamine 0.0005% (OPTI-FREE Express), Polyquaternium-1 0.001% with	*Staphylococcus aureus* (6%) *Staphylococcus epidermidis* (56%) *Staphylococcus saprophyticus* (45%)	*Delftia acidovorans* (5%) *Serratia marcescens* (5%) *Stenotrophomonas maltophilia* (11%)	Fungi	*Acantham oeba*

		myristamindopropyl dimethylamine 0.0005% (OPTI-FREE ReplineSH) - based disinfecting systems				
(Wu *et al.*, 2010)	64 asymptomatic lens wearers	Polyhexanide 0.0001% (AQuify), Hydrogen peroxide (CLEAR CARE), Polyquaternium-1 0.001% with myristamindopropyl dimethylamine 0.0005% (OPTI-FREE Express), Polyquaternium-1 0.001% with myristamindopropyl dimethylamine 0.0005% (OPTI-FREE ReplineSH), Hydrogen peroxide ('one step'; Aosept) , Hydrogen peroxide ('two step'; Oxysept 1 and 2) Chlorhexidine (Boston Lens) Polyhexanide 0.001% (Solocare) – based disinfecting systems	Coagulase-negative staphylococci (89%) *Bacillus* spp. (61%) *Micrococcus* spp. (44%) *Propionibacterium acnes* (28%)	*Serratia marcescens* (17%) *Achromobacter xyloxidans* (17%)	Filamentary fungi	NR
(Wiley *et al.*, 2012)	28 microbial keratitis patients (Mild keratitis: 5, Keratitis with focal infiltrates: 8, Corneal ulcer: 4, Asymptomatic control: 9)	NR	*Delftia* spp. (41%) (Identified by 16s RNA gene sequence analysis)	*Delftia* (41%) *Stenotrophomonas* (43%) *Achromobacter* (57%)	NR	NR

(Üstüntürk & Zeybek, 2012)	50 asymptomatic contact lens wearers	NR	Not isolated	Gram negative rods (40%) *Pseudomonas aeruginosa* (4%)	Fungi (36%)	Not detected
(Kilvington *et al*., 2012)	18 lens cases of corneal infiltrative events	NR	Not isolated	*Achromobacter* spp. (56%) *Stenotrophomonas maltophilia* (22%), *Serratia marcescens* (17%), *Delftia* spp. (11%) *Elizabethkingia* spp. (33%)	NR	NR
(Thakur & Gaikwad, 2014)	50 asymptomatic lens wearers	NR	*Staphylococcus aureus* (21%) *Bacillus* spp. (9.5%) Coagulase-negative staphylococci (2.5%) *Micrococcus* spp. (0.5%)	*Pseudomonas aeruginosa* (19.5%) *Klebsiella* spp. (5%) *Escherichia coli* (1%)	NR	NR
(Panthi *et al*., 2014)	46 asymptomatic lens wearers	Hydroxypropyl methylcellulose with EDTA 0.1% (Purecon-Puresoft), Dymed 0.0001%, EDTA 0.1% (Renu Multiplus), Polyquaternium-1 0.001% with myristamindopropyl dimethylamine 0.0005% (OPTI-FREE Express) – based disinfecting systems	*Staphylococcus aureus* (37%) *Staphylococcus epidermidis* (4.3%)	*Pseudomonas aeruginosa* (2%) *Escherichia coli* (9%)	NR	NR
(Szczotka-Flynn *et al*., 2014)	218 asymptomatic wears (cohort study); 1230 lens cases were cultured with no CIEs events	Hydrogen peroxide, Multipurpose disinfecting solution- based disinfecting system	Coagulase-negative staphylococci (10%) *Staphylococcus aureus* (10%) *Bacillus* spp. (3%)	*Pseudomonas aeruginosa* (3%)	Yeast (1%)	NR

1.4 CONTACT LENS INDUCED MICROBIAL KERATITIS

Contact lenses are usually considered safe, however they, sometimes cause contact lens induced corneal infections. Microbial keratitis (Willcox & Holden, 2001) whilst rare, is a serious potentially blinding corneal infection (Stapleton *et al*., 2008; Wihelmus *et al*., 1987). Microbial keratitis is characterized by an underlying inflammatory infiltrate in the corneal stroma with an overlying epithelial defect (Cheng *et al*., 1999; Edwards *et al*., 2009; Keay *et al*., 2006; Stapleton *et al*., 1993; Sweeney *et al*., 2003; Willcox *et al*., 2004). Contact lens related microbial keratitis can result in vision loss as a consequence of corneal scarring and perforation (Cheng *et al*., 1999; Stapleton *et al*., 2008; Wihelmus, 1987).

Depending on the study design and location, contact lens wear accounts for approximately 12% to 66% of all microbial keratitis events (Bourcier *et al*., 2003; Fong *et al*., 2004; Gebauer *et al*., 1996; Keay *et al*., 2006; Lam *et al*., 2002; Rattanatam *et al*., 2001; Schein *et al*., 2005; Wong *et al*., 2003). Published rates for the incidence of contact lens induced microbial keratitis are 2.2 - 4.1 per 10,000 wearers of soft daily lens wearers, and 13.3 - 20.6 per 10,000 wearers of soft extended wearers (Cheng *et al*., 1999; Lam *et al*., 2002; Poggio *et al*., 1989; Seal *et al*., 1999). For contemporary contact lens types, the annual incidence of microbial keratitis is 1.9 - 2.2 per 10,000 wearers of soft hydrogel daily wear lenses, 11.9 per 10,000 for silicone hydrogel daily wear, 19.5 per 10,000 for soft hydrogel extended wear and 25.4 per 10,000 for silicone hydrogel extended wearers (Stapleton *et al*., 2008). The risk of developing contact lens induced microbial keratitis is 9 to 15 times higher in extended lens wear compared to daily wear (Dart *et al*., 2008; Schein *et al*., 1989; Schein *et al*.,

2005; Stapleton *et al*., 2008). Silicon hydrogel contact lenses were introduced to the market to reduce hypoxia related corneal complications, but they have not influenced the incidence of microbial keratitis (Schein *et al*., 2005; Stapleton *et al*., 2008). **Table 1.2** summarises the incidence of microbial keratitis among the contact lens wearers from different studies, geographies, and lens types.

Multiple genera and species of microorganisms can be isolated from contact lens induced microbial keratitis. Bacteria predominate (77%) (Dart *et al*., 2008; Edwards *et al*., 2009; Erie *et al*., 1993; Galentine *et al*., 1984; Lam *et al*., 2002; Moriyama & Hofling-Lima, 2008; Stapleton *et al*., 2007; Meulen *et al*., 2008), followed by protozoa (20%) (Johnston *et al*., 2009; Stapleton *et al*., 2007) and fungi (5%) (Lam *et al*., 2002; Schein *et al*., 1989; Stapleton *et al*., 2007; Wilhelmus, 2001; Wilhelmus *et al*., 1988a). The most commonly isolated microbe from microbial keratitis is *Pseudomonas aeruginosa* (Donzis *et al*., 1987; Galentine *et al*., 1984; Stapleton *et al*., 2007; Wilson *et al*., 1990). *Acanthamoeba* species (Bennett *et al*., 1998; Cheng *et al*., 1999; Galentine *et al*., 1984; Mubareka *et al*., 2006; Stapleton *et al*., 2007) and fungi specially *Fusarium* spp. *Aspergillus* spp. and *Candida* spp. have also been isolated from contact lens related microbial keratitis but at a much lower frequency than bacteria (Stapleton *et al*., 2007; Wilhelmus *et al*., 1988b). **Table 1.3** details the variety of microorganisms isolated from microbial keratitis in various studies.

Table 1.2: Summary of the studies reporting the incidence of microbial keratitis in contact lens wearers.

Study (Geography)	Type of disease condition	Sample size	Case capture	Type of lens and wearing schedule	Incidence per 10,000 (95% CI)		
					Soft daily	Soft extended	RGP Daily
(Poggio *et al.*, 1989) (USA)[a]	Microbial keratitis	195 contact lens induced ulcerative keratitis was reported	Four months prospective surveillance of all practicing ophthalmologists	Soft daily wear	4.1 (2.9-5.2)	20.9 (15.1 – 26.7)	4.0 (0- 8.2)
(MacRae *et al.*, 1991) (USA)[c]	Corneal ulcer	22,739 contact lens wearers were studied	Prospective, clinical data analysed for 1980-1988	Soft daily wear	5.2 (0-15.4)	18.0 (8.2-27.8)	-
(Nilsson & Montan, 1994) (Sweden)[b]	Contact lens induced keratitis	440, 000 contact lens wearers were studied	Three months prospective national surveillance of all ophthalmologists	Soft daily wear	2.2 (0.4-3.9	13.3 (4.1 – 22.6)	-
(Stevenson & Seal, 1998) (Scotland)[c]	Presumed microbial keratitis	6750 soft CL wearers with microbial keratitis	Eight months (1994-1995) prospective, population surveillance via 8 hospitals	Different types	2.7 (1.6-3.7)	-	-

(Cheng *et al.*, 1999) (Netherlands)[a]	Presumed microbial keratitis	92 CL wearers with microbial keratitis	Three months prospective surveillance of all practicing ophthalmologists	Soft daily and extended wear	3.5 (2.7-4.5)	20.0 (10.3-35.0)	1.1 (0.6-1.7)
(Lam *et al.*, 2002) (Hong Kong)[b]	Presumed microbial keratitis	223 CL induced microbial keratitis	17-months prospective survey of 2 hospitals and 27 private ophthalmologists	Soft daily wear	3.1 (2.1-4.0)	-	-
(Schein *et al.*, 2005) (USA)[c]	Presumed microbial keratitis	5561 Contact lens wearers (Total surveyed 6245)	12-month prospective, cohort post market widely distributed 10 cases and symptoms and review surveillance study	Soft extended wear lenses	-	39.7(12.7 – 92.8)	-
(Stapleton *et al.*, 2008) (Australia)[a]	Microbial keratitis	285 cases	Prospective, 12 months, population-based surveillance study	Soft hydrogel, silicon hydrogel and rigid lens wearers	1.9 (1.8-2.0)	11.9 (10.0-14.6)	1.2 (1.1-1.5)

a, denominator derived from the random telephone survey of the community.
b, denominator derived from fitting surveys.
c, denominator derived from the number of contact lens wearers.

Table 1.3: Microorganisms isolated from microbial keratitis induced by contact lens wear (Adapted and updated from Willcox *et al*. 2001b).

Microorganisms reported Bacteria (Gram negative)	**Authors**
Achromobacter xyloxidans	(Moriyama & Hofling-Lima, 2008)
Acinetobacter calcoaceticus	(Cheng *et al*., 1999), (Schein *et al*., 1989)
Acinetobacter spp.	(Houang, Lam, Fan, & Seal, 2001), (Bennett *et al*., 1998), (Ormerod & Smith, 1986)
Alcaligenes spp.	(Moriyama & Hofling-Lima, 2008), (Bharathi *et al*., 2007)
Citrobacter freundii	(Moriyama & Hofling-Lima, 2008)
Enterobacter aerogenes	(Cooper & Constable, 1977), (Moriyama & Hofling-Lima, 2008)
Enterobacter spp.	(Cheng *et al*., 1999), (Bharathi *et al*., 2007)
Escherichia coli	(Houang *et al*., 2001),(Cheng *et al*., 1999), (Weissman *et al*., 1984)
Haemophilus influenzae	(Al-Yousuf *et al*., 2009), (Mondino *et al*., 1986)
Kingella kingae	(Otri *et al*., 2013)
Klebsiella oxytoca	(Dart *et al*., 1988), (Lemp, Blackman, Wilson, & Leveille, 1984), (Schein *et al*., 1989)
Klebsiella oxygenate	(Stapleton *et al*., 2007), (Stapleton *et al*., 2017)
Klebsiella pneumoniae	(Schein *et al*., 1989)
Klebsiella spp.	(Cheng *et al*., 1999), (Moriyama & Hofling-Lima, 2008), (Bharathi *et al*., 2007)
Moraxella lacunata	(Dart *et al*., 1988)
Moraxella spp.	(Schein *et al*., 1989), (Stapleton *et al*., 1993), (Moriyama & Hofling-Lima, 2008)
Morganella morganii	(Mondino *et al*., 1986), (Schein *et al*., 1989)
Nocardia spp.	(Stapleton *et al*., 2017)
Proteus mirabilis	(Alfonso *et al*., 1986), (Schein *et al*., 1989), (Moriyama & Hofling-Lima, 2008)
Proteus morganii	(Mondino *et al*., 1986)
Proteus vulgaris	(Patrinely, Wilhelmus, Rubin, & Key, 1985)
Pseudomonas cepacia	(Patrinely *et al*., 1985)
Pseudomonas aeruginosa	(Otri *et al*., 2013), (Yousuf *et al*., 2009), (Green *et al*., 2008), (Yu *et al*., 2007), (Houang *et al*., 2001), (Ormerod & Smith, 1986), (Lam *et al*., 2002), (Stapleton *et al*., 1995), (Ormerod & Smith, 1986), (Bennett *et al*., 1998), (Stapleton *et al*., 2007), (Moriyama & Hofling-Lima, 2008), (Bharathi *et al*., 2007), (Stapleton *et al*., 2017)
Pseudomonas fluorescens	(Stapleton *et al*., 1995), (Moriyama & Hofling-Lima, 2008)
Pseudomonas spp.	(Galentine *et al*., 1984)(Sharma *et al*., 2003), (Houang *et al*., 2001), (Stapleton *et al*., 1993), (Alfonso *et al*., 1986), (Stapleton *et al*., 2007), (Dejaco-Ruhswurm *et al*., 2001)

Serratia liquefaciens	(Galentine *et al*., 1984), (Stapleton *et al*., 2007)
Serratia marcescens	(Yu *et al*., 2007), (Alfonso *et al*., 1986), (Schein *et al*., 1989), (Cheng *et al*., 1999), (Cohen *et al*., 1987), (Dart *et al*., 1988), (Stapleton *et al*., 2007), (Stapleton *et al*., 2017)
Serratia spp.	(Houang *et al*., 2001), (Ormerod & Smith, 1986), (Stapleton *et al*., 2007), (Moriyama & Hofling-Lima, 2008), (Bharathi *et al*., 2007)
Stenotrophomonas maltophilia	(Houang *et al*., 2001), (Cheng *et al*., 1999)
Bacteria (Gram positive)	
Aerobic spore-forming bacilli	(Cheng *et al*., 1999)
Alpha-haemolytic *streptococci*	(Sharma *et al*., 2003), (Dart, 1988), (Ormerod & Smith, 1986), (Bennett *et al*., 1998)
Bacillus cereus	(Patrinely *et al*., 1985), (Stapleton *et al*., 2007)
Bacillus spp.	(Ormerod & Smith, 1986)
Coagulase-negative staphylococci	(Schein *et al*., 1989), (Green *et al*., 2008), (Moriyama & Hofling-Lima, 2008), (Houang *et al*., 2001), (Bennett *et al*., 1998), (Stapleton *et al*., 2017)
Corynebacterium diphtheriae	(Cheng *et al*., 1999)
Diphtheroids	(Houang *et al*., 2001), (Cohen *et al*., 1987), (Dunn *et al*., 1989)
Micrococcus spp.	(Ormerod & Smith, 1986)
Nocardia spp.	(Houang *et al*., 2001), (Weissman *et al*., 1984), (Stapleton *et al*., 2007)
Propionibacterium acnes	(Mondino *et al*., 1986), (Weissman *et al*., 1984), (Dunn *et al*., 1989)
Staphylococcus aureus	(Otri *et al*., 2013), (Green *et al*., 2008), (Green *et al*., 2008), (Moriyama & Hofling-Lima, 2008), (Ormerod & Smith, 1986), (Bennett *et al*., 1998), (Cohen *et al*., 1987), (Mondino *et al*., 1986), (Patrinely *et al*., 1985), (Sharma *et al*., 2003), (Weissman *et al*., 1984), (Stapleton *et al*., 2017)
Staphylococcus epidermidis	(Ormerod & Smith, 1986), (Cohen *et al*., 1987), (Dart, 1988), (Mondino *et al*., 1986), (Patrinely *et al*., 1985), (Sharma *et al*., 2003), (Stapleton *et al*., 2007)
Staphylococcus spp.	(Galentine *et al*., 1984), (Al-Yousuf, 2009), (Cheng *et al*., 1999), (Cohen *et al*., 1987), (Stapleton & Dart, 1993), (Stapleton *et al*., 2007), (Moriyama & Hofling-Lima, 2008),
Streptococcus pneumoniae	(Galentine *et al*., 1984), (Stapleton *et al*., 2007), (Green *et al*., 2008), (Green *et al*., 2008), (Bennett *et al*., 1998), (Dart, 1988), (Stapleton *et al*., 2017)
Streptococcus spp.	(Al-Yousuf, 2009), (Alfonso *et al*., 1986),

	(Cheng *et al*., 1999), (Galentine *et al*., 1984)
Fungi	
Acremonium spp.	(Stapleton *et al*., 2017)
Aspergillus flavus	(Wong *et al*., 1997)
Arthographis karlae	(Perlman & Binns, 1997)
Aspergillus wentii	(Wilhelmus *et al*., 1988b)
Aureobasidium pulluans	(Moriyama & Hofling-Lima, 2008)
Candida parapsilosis	(Wilhelmus *et al*., 1988b)
Candida spp.	(Patel & Hammersmith, 2008), (Wilhelmus *et al*., 1988b), (Schein *et al*., 1989), (Galentine *et al*., 1984), (Moriyama & Hofling-Lima, 2008), (Stapleton *et al*., 2017)
Candida tropicalis	(Wilhelmus *et al*., 1988b)
Cephalosporium spp.	(Wilhelmus *et al*., 1988b)
Fusarium dimerum	(Stapleton *et al*., 2017)
Fusarium solani	(Patel & Hammersmith, 2008), (Green *et al*., 2008), (Green *et al*., 2008), (Wilhelmus *et al*., 1988b)
Fusarium spp.	(Tu & Joslin, 2010), (Patel & Hammersmith, 2008), (Rao *et al*., 2007), (Gorscak *et al*., 2007), (Matthew Green *et al*., 2008), (Alfonso *et al*., 1986)
Paecilomyces spp.	(Wilhelmus *et al*., 1988b)
Penicillium spp.	(Ormerod & Smith, 1986)
Trichosporon mucoides	(Stapleton *et al*., 2017)
Protozoa	
Acanthamoeba spp.	(Schein *et al*., 1989), (Tu & Joslin, 2010), (Otri *et al*., 2013), (Yoder *et al*., 2012), (Al-Yousuf, 2009), (Houang *et al*., 2001), (Bennett *et al*., 1998), (Cohen *et al*., 1987), (Sharma *et al*., 2003), (Stapleton & Dart, 1993), (Stapleton *et al*., 2007), (Moriyama & Hofling-Lima, 2008), (Dejaco-Ruhswurm *et al*., 2001), (Stapleton *et al*., 2017)
Vahlkampfia spp.	(Aitken *et al*., 1996; Bennett *et al*., 1998)
Hartmanella spp.	(Aitken *et al*., 1996)
Naegleria spp.	(Larkin *et al*., 1990)

1.4.1 Bacterial Keratitis

Approximately 70% to 80% of contact lens induced microbial keratitis are associated with bacteria, predominantly with Gram negative bacteria (Dart *et al*., 1991; Erie *et al*., 1993; Stapleton *et al*., 2007). The bacteriological profile in keratitis shows large disparities between populations living in developed or in developing countries. These variations could be due to the fact that less industrialized countries have significantly lower number of contact lens wearers, hence fewer contact lens related infections.

Bacterial keratitis in CL wearers is mostly associated with gram-negative bacteria and *P. aeruginosa* is the most common bacteria, (Lam *et al*., 2002; Schein *et al*., 2005; Stapleton *et al*., 2007, 1995) isolated form the site of ocular infections in up to 70% of the contact lens induced corneal infections (Alfonso *et al*., 1986; Holland *et al*., 1993). *P. aeruginosa* keratitis rapidly progresses with the destruction of the cornea, and eventually can lead to corneal scarring and vision loss (Matsumoto, 2004; Stapleton *et al*., 1995). The strong association between *P. aeruginosa* and contact lens associated corneal infection is intriguing (Stapleton *et al*., 1995). The contact lens, lens storage case and ocular environment may offer a suitable environment for the survival of environmental bacteria. *P. aeruginosa* can adhere and colonize lens materials during use and survive in lens storage cases, due to its resistance to contact lens care systems (Lakkis & Fleiszig, 2001).

Other commonly isolated Gram negative bacteria includes *Serratia marcescens*, (Cheng *et al*., 1999; Ormerod & Smith, 1986) *Acinetobacter* spp. and *Klebsiella* spp. (Dart *et al*., 2008; Edwards *et al*., 2009; Erie *et al*., 1993; Lam *et al*., 2002; Moriyama & Hofling-Lima, 2008; Stapleton *et al*., 2007; Meulen *et al*., 2008). *Serratia*

marcescens can be associated with both severe (Lazachek, 1971) and mild keratitis (Lass *et al*., 1981).

Gram positive bacteria are also isolated from contact lens induced bacterial keratitis. Bourcier *et al.* showed low level of Gram negative bacteria isolated from contact lens induced microbial keratitis, while two third of the bacterial keratitis in contact lens wear was associated with Gram positive bacterial species including streptococci and staphylococci, particularly with *S. aureus* (Bourcier *et al*., 2003).

1.4.2 Fungal Keratitis

Fungal keratitis in contact lens wear is a rare phenomenon, which occurs in less than 5% of contact lens related microbial keratitis (Lam *et al*., 2002; Schein *et al*., 1989; Stapleton *et al*., 2007; Wilhelmus, 2001; Wilhelmus *et al*., 1988a). Fungal keratitis can be associated with different climatic conditions and agrarian populations; i.e. in India, 35% to 50% of microbial keratitis is associated with fungal keratitis (Srinivasan, Mascarenhas, & Prashanth, 2008). Fungal keratitis, it has received significant attention in the past few decades due to an increased rate of *Fusarium* keratitis among lens wearers, initially in Singapore (2005 and 2006) and then in Hong Kong (Ma *et al*., 2009), the United States of America and France (Gaujoux *et al*., 2008).

In Singapore, from 2005 to 2006, the rate of contact lens induced fungal keratitis was 2.35 case per 10,000 contact lens wearers. The increased rate in Singapore and elsewhere was associated with using ReNu® MoistureLoc™ brand contact lens cleaning and disinfecting solutions (Khor *et al*., 2006). When checked retrospectively, the unopened ReNu® MoistureLoc™ bottles were not contaminated with fungi. Alexidine and polyquaternium-10 was present in this formulation of ReNu® MoistureLoc™. Under certain non-compliant usages (irregular changing of solution,

not recapping the lens case and solution bottle between use), ReNu® MoistureLoc™ showed reduced biocidal efficacy which may have caused Fusarium contamination (Khor *et al*., 2006; Levy *et al*., 2006; Zhang *et al*., 2006). This led to ReNu® MoistureLoc™ solution being permanently withdrawn from the market globally in May 2006. Gower *et al*., reported a significant reduction in the number of fungal keratitis cases, from the tertiary eye care centres across the United States over a 7-year period (2001 to 2007) after the removal of ReNu® MoistureLoc™ from sale (Gower *et al*., 2010). **Table 1.4** summarizes the risk factors and the types of fungi species isolated from fungal keratitis.

Table 1.4: Summary of reported Fusarium keratitis and the risk associated with contact lens wearers and the isolated fungi species.

Study	Year, location	Sample size	Case capture	Sources (Site of the sample collection)	Risk associated with Cl wear (N, %)	Isolated microorganisms
(Chander & Sharma, 1994)	Northern India	730 patients	from 1 January 1987 to 31 December 1992	Corneal scrape	Associated with contact lens wear (15, 24%)	*Aspergillus* spp. (40%) *Fusarium* sp. (16%) *Aspergillus fumigatus* (15%) *Aspergillus flavus* (14%) *Candida albicans* (8%) *Curvularia* sp. (5%)
(Tanure *et al*., 2000)	Philadelphia, Pennsylvania, USA	24 eyes with keratitis	Retrospective study (from January 1991 to March 1999)	Corneal scrape	Associated with contact lens wear (30%), Extended wear SCL (13%), Daily wear (8%)	*Candida albicans* (46%) *Fusarium* sp. (25%) *Scedosporium apiospermum* (8%)
(Alfonso *et al*., 2006b)	Miami, USA	122 patients	Retrospective review of microbiologic records from January 1, 2004, through April 15, 2006	Corneal scrape	Associated with contact lens wear (26, 21%)	*Fusarium* sp. (54%) *Fusarium oxysporum* (69%) *Fusarium solani* (10%)
(Khor *et al*., 2006)	Singapore	66 patients with 68 affected eyes	March 2005 through May 2006	Corneal scrape or corneal specimen, contact lens and contact	Associated with contact lens (10%), contact lens case (4%)	*Fusarium* sp. (85%)

				lens cases		
(Ng *et al*., 2008)	Hong Kong	16 patients	Retrospective case review (From July 2005 to June 2006)	Corneal scrape and contact lens solution	Two case reports	*Fusarium* sp. (44%) *Fusarium oxysporum* (19%) Not specified (36%)
(Keay *et al*., 2011)	USA	733 patients	Retrospective review (January 1, 2001, and December 31, 2007)	Corneal scraping or biopsies	Associated with contact lens wear (268, 37%)	Filamentous fungi (83%) Yeast (53%)
(Nielsen *et al*., 2015)	Aarhus and Copenhagen, Denmark	25 patients	from 2000 to July 2013	Smear (60%), scrape (16%), biopsy (12%) or corneal buttons after keratoplasty (12%).	Associated with contact lens wear (23%)	52% with *Candida*, 20% with *Fusarium*, 16% with *Aspergillus* 12% with mixed filamentous fungi.

1.4.3 Acanthamoeba Keratitis

Acanthamoeba is the most commonly isolated protozoan from contact lens induced keratitis (Butler *et al*., 2005; Moriyama & Hofling-Lima, 2008). *Acanthamoeba* keratitis is a painful, potentially blinding corneal infection and may affect both eyes (Badenoch, 2010; Panjwani, 2011; Roters *et al*., 2001).

Acanthamoeba keratitis was first reported in 1970*s*. Radford *et al*. reported the annual incidence of *Acanthamoeba* keratitis for the 2 years from 1 October 1997 to 30 September 1999 was 1.13 to 1.26 per million adults and, for contact lens wearers, 21.14 and 17.53 per million. Up to 93% of *Acanthamoeba* keratitis cases are associated with contact lens wear (Nagington *et al*., 1974; Radford *et al*., 2002), and predominantly with daily soft contact lens wearers.

There was a global outbreak of *Acanthamoeba* keratitis in 2007 associated with the use of MoisturePlus™ MPDS (González-Méijome *et al*., 2007; Joslin *et al*., 2007). However, unlike with Fusarium keratitis and MoistureLoc™, the number of *Acanthamoeba* keratitis cases that occur in the USA has remained elevated even after the removal of MoisturePlus™ from sale (Carnt & Stapleton, 2016; Johnston *et al*., 2009; Tu & Joslin, 2010).

The most commonly isolated *Acanthamoeba* species are *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* (Al-Yousuf, 2009; Bennett *et al*., 1998; Cohen *et al*., 1987; Houang *et al*., 2001; Otri *et al*., 2013; Tu & Joslin, 2010; Yoder *et al*., 2012). Other protozoa involved in contact lens induced keratitis include *Naegleria* spp. (Aitken *et al*., 1996; Bennett *et al*., 1998), *Vahlkampfia* spp. (Aitken *et al*., 1996), and *Hartmannella* spp. (Gray *et al*., 1995; Larkin *et al*., 1990).

Major historical risk factors associated with *Acanthamoeba* keratitis include: the use of homemade saline, swimming/showering/bathing with lenses, irregular disinfection of lenses and rinsing contact lens and lens storage cases with tap water (especially from roof top storage tanks) (Radford *et al*., 1995; Seal *et al*., 1999). Additionally, a combination of factors such as the use of tap water to clean the contact lens or lens storage cases and the non-compliant hygiene behaviour of contact lens wearers during hand washing may result in a high risk of developing *Acanthamoeba* keratitis (Bonilla-Lemus *et al*., 2010; Kilvington, 2004; Uno *et al*., 2011).

1.5 CONTACT LENS INDUCED INFLAMMATION:

Microbial contamination of contact lenses can cause corneal inflammatory conditions such as contact lens induced acute red eye (CLARE), contact lens induced peripheral ulcer (CLPU) and infiltrative keratitis (IK) (Bates *et al*., 1989; Holden *et al*., 1996; Sweeney *et al*., 2003; Willcox *et al*., 2010; Willcox *et al*., 2004).

1.5.1 Contact Lens-induced Acute Red Eye (CLARE)

Contact lens induced acute red eye is by definition always associated with overnight lens wear (Sweeney *et al*., 2003a; Szczotka-Flynn *et al*., 2010). Contact lens induced acute red eye has been reported to occur in 33% of soft lens wearers and 34% of continuous lens wearers (Nilsson, 2001). Contact lens induced acute red eye is most commonly observed during the first 3 months of extended wear, although it can occur at any time (Nilsson, 2001; Sankaridurg *et al*., 2000). Risk factors associated with CLARE include: high water content lenses, a tight fitting lens and contact lens users who suffer from respiratory tract infection (Sankaridurg *et al*., 1996). Contact lens induced acute red eye is characterized by severe conjunctival, especially circumlimbal hyperaemia (Sweeney *et al*., 2003; Willcox *et al*., 2004) and diffuse corneal infiltrates.

Gram negative bacteria commonly associated with CLARE include *Haemophilus influenzae, Haemophilus parainfluenzae, S. marcescens* and *Pseudomonas* spp. (Sankaridurg *et al*., 2004; Sankaridurg *et al*., 1996; Willcox *et al*., 2004). Occasionally with Gram positive bacteria such as *Streptococcus pneumoniae* (Sankaridurg *et al*., 1999) can be isolated from cases of CLARE.

1.5.2 Contact Lens Peripheral Ulcer (CLPU)

Contact lens induced peripheral ulcer can occur during extended or daily wear of lenses (Grant *et al*., 1998; Long *et al*., 2011; Iruzubieta *et al*., 2001a; Sankaridurg *et al*., 2004). Contact lens induced peripheral ulcer is characterized by a small circular full-thickness epithelial lesion in the peripheral cornea, associated with stromal infiltration (Grant *et al*., 1998; Sankaridurg *et al*., 1999; Sankaridurg *et al*., 2000).

The incidence of CLPU varies from 0.3% to 13.6%, depending on the study location and the sample population (Long *et al*., 2011; Iruzubieta *et al*., 2001b; Sankaridurg *et al*., 1999, 2004). Among extended lens wearers, the rate of CLPU varies between 1.6% to 2.9% in Australia (Zantos & Holden, 1978) and up to 13% per year in India.(Sankaridurg *et al*., 1999). Most cases of CLPU are associated with low numbers of Gram positive bacteria such as *S. aureus* and *S. epidermidis* on contact lenses or on the lid margins (Jalbert *et al*., 2000; Szczotka-Flynn *et al*., 2009; Willcox *et al*., 2011).

1.5.3 Infiltrative Keratitis (IK)

Infiltrative keratitis is characterised by focal or multiple infiltrates in the cornea, with no overlying epithelial break. This condition is commonly symptomatic – although the severity of the symptoms can vary (Szczotka-Flynn *et al*., 2009). The annual incidence of IK is 2.1% to 17.8 per 100 eyes (Sankaridurg *et al*., 2004).

Infiltrative keratitis is associated with contamination by both Gram positive and Gram negative bacteria (Szczotka-Flynn *et al*., 2009). Thirty percent of IK cases showed clinical features resembling CLARE, but IK is differentiated from CLARE as the symptoms can occur at any time during lens wear (not during or immediately after a period of sleep) (Willcox *et al*., 2004).

1.6 FACTORS ASSOCIATED WITH CONTACT LENS CASE CONTAMINATION:

Inadequate cleaning of contact lens cases, occurs in 72% of the asymptomatic lens wearers (Stapleton & Wu, 2011a; Wu *et al*., 2015). Other factors including insufficient hand washing before handling the lens case, inappropriate cleaning instructions provided by the practitioner's regarding the storage environment of the lens case, air drying and general habits may also be found responsible for lens case contamination (Wu *et al*., 2011b).

1.6.1 Hand Washing:

While hand washing is a risk factor in contact lens related adverse events, there is limited evidence to suggest if hand washing is strongly associated with case contamination. Wu *et al*. demonstrated that contact lens case contamination is often associated with inappropriate handwashing in asymptomatic lens wearers in a community study (Wu *et al*., 2015). Only 27% of contact lens wearers have reported an awareness that hands hygiene was an important factor in avoiding contact lens related adverse events (Donshik *et al*., 2007). However, the result appears to be odd that 88% of the lens wearers carry the hand washing step prior to handling contact lens which may not be as effective to keep the contact lens or contact lens cases free of microbial contamination (Wu *et al*., 2010). Poor hand washing is common in 14% to

50% of lens wearers and vary with the phrasing of questionnaire been used (Radford *et al*., 1993; Sokol *et al*., 1990; Yung *et al*., 2007).

In a case control study, more than one-third of contact lens wearers exhibited poor hand washing (not washing hands or only used tap water), and indeed, lens cases from these participants were significantly associated with the higher level of lens case contamination. Although, *in vitro* studies have demonstrated that rubbing lens cases is effective in removing contamination from the lens cases. However, rubbing the lens cases after inadequate hand washing had significantly higher levels of lens case contamination than those cases not being rubbed at all (Wu *et al*., 2010; Wu *et al*., 2010b). These findings support that hand washing may have impact on lens case contamination which needs further investigation to explore the association between hand washing and contact lens induced corneal infiltrative events.

1.6.2 Contact Lens Case Cleaning:

Inadequate cleaning of contact lens storage cases has a major impact on the lens case contamination during wear (Wu *et al*., 2011; Wu *et al*., 2015). However, current recommended lens case hygiene practices and good hygiene compliance do not necessarily ensure a lens case free of microbial contamination (Stapleton *et al*., 1995).

Variations in contact lens cleaning guidelines by different manufacturers demonstrated significant difference in the level of microbial contamination of lens cases (Wu *et al*., 2011; Wu *et al*., 2011b). Initially, the cleaning of contact lens cases with hot water and allowing them to air dry was the most efficient method of cleaning (Larragoiti *et al*., 1994). But later Larkin *et al*. discourage the use of water which could be the source of *Acanthamoeba* (Larkin *et al*., 1990) and can be the cause of severe ocular infections (Illingworth, 1998; Kilvington, 2004; Radford *et al*., 2002).

The FDA recommended guidelines are generally considered a standard guideline, and these include the steps of rubbing, rinsing (with disinfecting solution), and air drying the lens case face down after use ("FDA. Contact lens and care product guidance documents. FDA Executive Summary. Prepared for the meeting of the ophthalmic devices panel of the medical devices advisory committee; May 13, 2014. Silver Spring, MD, U.S. Food and Drug Administration, 2014. Ava," 2008). Manufacturers have occasionally modified the instructions provided by FDA for simplicity, such as the elimination of the rubbing step from the lens case cleaning protocol. Such modifications are inconsistent with previous *in vitro* studies showing digital "rub, rinse and tissue wiping" is the most effective cleaning recommendation in reducing biofilm formation (Wu *et al*., 2011a; Carnt *et al*., 2010; Wu *et al*., 2011b) offering support for the FDA guidelines (**Table 1.5)**.

Even the new generation silver-impregnated lens cases can benefit from the "rub, rinse and tissue wipe" regimen (Amos & George, 2006; Dantam *et al*., 2011). Recently a new warming device has been introduced (Willcox *et al*., 2012) which significantly reduced bacterial contamination when lens cases were left at 60ºC.

1.6.3 Air-drying of the Lens Cases:

Air drying of lens cases face down along with rubbing and cleaning with disinfecting solution, is recommended by the FDA ("FDA. Contact lens and care product guidance documents. FDA Executive Summary. Prepared for the meeting of the ophthalmic devices panel of the medical devices advisory committee; May 13, 2014. Silver Spring, MD, U.S. Food and Drug Administration, 2014. Ava," 2008). Among optometrists there is still a lot of disparity in the recommendations about the case drying (Wu *et al*., 2010). Studies have confirmed that the aerosol effect of toilet

flushing results in airborne bacteria and viruses which can settle down on the adjacent surfaces at considerable distance (Barker & Jones, 2005; Gerba *et al*., 1975). Wu *et al.* promoted air-drying of the lens case in a face down position in a non-humid environment to minimize the exposure of air-borne contaminants and to accelerate the air-drying process (Wu *et al*., 2010a). Conversely, silver-impregnated storage cases showed reduced bacterial contamination when stored wet compared to air drying or when lens cases were recapped (Dantam *et al*., 2011).

1.6.4 Contact Lens Case Replacement Schedule:

The irregular replacement of lens cases is a risk factor for contact lens related microbial keratitis. It is recommended that lens cases be replaced at frequent interval to avoid ocular infections, even if the contact lens cases are disinfected regularly (Stapleton *et al*., 2008). In community studies, a positive association was found between the age of the contact lens case and the level of microbial contamination (Devonshire *et al*., 1993; Wu *et al*., 2010). The FDA recommends lens cases to be replaced every three months ("FDA. Contact lens and care product guidance documents. FDA Executive Summary. Prepared for the meeting of the ophthalmic devices panel of the medical devices advisory committee; May 13, 2014. Silver Spring, MD, U.S. Food and Drug Administration, 2014. Ava," 2008), while manufacturers' recommend replacing lens cases every month or with every purchase of new solutions (Larkin *et al*., 1990; Pinna *et al*., 2004; Wu *et al*., 2010). A lower percentage of lens case contamination has been observed in lens cases that were used for less than 9 months (Wu *et al*., 2010) however, the prolonged use of lens cases is still commonly practiced (Hickson-Curran, Chalmers, & Riley, 2011). Most studies have considered lens cases contamination with conventional lens cases and no studies

have examined the replacement schedule for silver-impregnated lens cases (Amos & George, 2006; Dantam *et al*., 2012).

1.6.5 Contact Lens Storage Case Designs and Materials:

The design of the lens cases can influence the rate and degree of case contamination. Kanpolat *et al.* suggested that lens case should have an easily cleanable design to avoid biofilm formation (Kanpolat *et al*., 1992). The inner surface of the case wells of flat lens cases can be smooth or have ridges of varying numbers and depth, and this can affect the degree of microbial adhesion (Wu *et al*., 2015). Other factors such as surface area, polishing and the presence of cracks on the surface may provide an ideal niches for bacterial colonization (McLaughlin-Borlace *et al*., 1998). Also, it is difficult to clean at the peripheral edges of the lens cases and ridges are present at the upper peripheral rim might increase the chance of bacterial colonization. Wu *et al*. observed variations in the level of lens case contamination at different locations from the same lens case (Wu *et al*., 2010). *Micrococcus* spp. *Bacillus* spp. and coagulase-negative staphylococci were more commonly isolated from the lower inner base of the cases than the upper rim (Wu *et al*., 2010). Also mismatching lens case and MPDS can affect contact lens case contamination (Wu *et al*., 2015).

The surface material of contact lens storage cases may play an important role in bacterial adhesion. Factors such as chemical composition of material, surface charge, hydrophobicity and surface roughness are likely to influence initial microbial adhesion. Contact lens cases on the market are mostly made of polyethylene or polypropylene. However, often various parts of contact lens cases are made of different material; e.g. commercially available Complete EasyRub (Advanced Medical optics [AMO], Santa Ana, CA) cases have smoother (inner surface) case wells which are made from

acrylonitrile butadiene styrene while the lids are made from polypropylene. ReNu® MultiPlus® (Bausch & Lomb, Rochester New York) lens cases are moulded from polypropylene both for the case well and lids.

Variations in lens case materials may influence the adhesion of bacteria such as the marine *Pseudomonas* spp. adhere in higher numbers to hydrophobic polypropylene surfaces with little or more surface charge compared to other hydrophilic plastics (Teflon, Polyethylene, Polystyrene and Polyethylene terephthalate) (Fletcher & Loeb, 1979). Thus, the hydrophobic polypropylene surfaces of contact lens case may encourage bacterial adhesion on lens cases (Willcox *et al*., 2010).

However, there is limited evidence in support of a relationship between lens case designs and materials and the rate of lens case contamination *in vivo*, which may be worthwhile to consider in future study.

Table 1.5: Contact lens case maintenance protocol, based on the FDA guidelines (FDA, 2008)

Summary of contact lens storage case maintenance technique
The summary of a uniform and effective instruction to lens wearers for contact lens case cleaning and maintenance by eye-care practitioner.
1. Hands should always be washed thoroughly before touching eyes and handling contact lens storage cases. 2. Use of soap is recommended for hand washing with vigorous rubbing and rinsing with water. 3. Hands should be dried using a single use towel or tissue paper to reduce the germs on the skin including those that are found in tap water. 4. The left-over contact lens solution should be emptied after each use. 5. Contact lens cases should not be exposed to any water (distilled water, tap water or any homemade saline solution) which may be associated with *Acanthamoeba* keratitis, a corneal infection that is resistant to treatment and cure. 6. Contact lens and lens case cleaning and disinfecting solution should be used based on optometrist's recommendation. 7. Lens storage case should be rubbed after instillation of disinfection solution, at least for 10 seconds including the base of the case well and the rims. Contact lens case lids are also needed to clean thoroughly. 8. Lens storage case should be thoroughly rinsed with recommended solution at least for 10 seconds. 9. Lens case needs to dry with lint free tissue paper. 10. Lens case also can be air-dried by face down in clean and non-humid environment. 11. Above mentioned contact lens storage cases cleaning and disinfection steps should follow at every occasion of lens removal. 12. Contact lens storage case needs to replace within 3 months.
Note: - The effect of temperature of contact lens case drying is different based on the type of designed cases. Over that it needs to find the proper hygiene guidelines for anti-microbial surface coated contact lenses. Recapping the contact lens case needs further investigations, particularly in silver-impregnated contact lens storage cases.

1.6.6 Factors associated with Contact Lens Induced Microbial Complications

Overnight use of daily wear lenses (Dart *et al*., 1991; Lam *et al*., 2002), longer duration in extended wear (Cheng *et al*., 1999; Dart *et al*., 1991; Lam *et al*., 2002; Schein *et al*., 1989), irregular replacement of contact lenses cases (Stapleton *et al*., 2012), poor contact lens hygiene and compliance (Stapleton *et al*., 2012), general hygiene (Dart *et al*., 1991; Morgan *et al*., 2005; Poggio *et al*., 1989) and gender (Dart *et al*., 1991; Poggio *et al*., 1989; Stapleton *et al*., 1993) are frequently associated with an increased risk of contact lens induced microbial keratitis.

Overnight use of contact lens was the most commonly associated with contact lens induced corneal infiltrative events (Chalmers *et al*., 2011; Morgan *et al*., 2005). Other risk factors include the working environment, smoking (Chalmers *et al*., 2007; Chalmers *et al*., 2011; Cutter *et al*., 1996; McNally *et al*., 2003), age (Chalmers *et al*., 2007; Chalmers *et al*., 2011; McNally *et al*., 2003) and the history of prior contact lens related corneal inflammatory conditions (McNally *et al*., 2003; Szczotka-Flynn *et al*., 2007). However, the microbial contamination of contact lens cases also can occur in asymptomatic lens wear (Wu *et al*., 2010). Inadequate cleaning of lens cases, air-drying of the lens cases during storage and unhygienic storing of lens cases were the commonly found factors related to contact storage cases hygiene significantly associated with lens case contamination in asymptomatic lens wear (**Table 1.6**).

Table 1.6: Factors associated with contact lens case contamination in asymptomatic contact lens wear.

Category of risk factors		Associated factors	Asymptomatic
Contact lens related		Lens wearing experience	(Wu *et al*., 2015)
		Contact lens replacement schedule	(Yung *et al*., 2007)*, (Devonshire *et al*., 1993)*
		Duration of lens wear	(Yung *et al*., 2007)*
		Type of contact lens	(Devonshire *et al*., 1993)*
Contact lens storage case associated		Rubbing of lens case	(Wu *et al*., 2015)*
		Amount of disinfecting solution used in lens cases	(Wu *et al*., 2015)
		Frequency of lens case replacement/ Age of lens cases	(Wu *et al*., 2010)*, (Wu *et al*., 2015), (Yung *et al*., 2007), (Devonshire *et al*., 1993)*
		Poor case hygiene	(Wu *et al*., 2010), (Wu *et al*., 2015)
		Air-drying of lens cases	(Wu *et al*., 2015)*
		Storing/airdrying the cases in unhygienic places	(Wu *et al*., 2011), (Wu *et al*., 2015)*
		Manufactures or practitioners recommended compliance	(Wu *et al*., 2011)
Care system related		Type of disinfecting system used	(Devonshire *et al*., 1993), (Wu *et al*., 2015), (Yung *et al*., 2007)*, (Jiang *et al*., 2014)*, (Dantam *et al*., 2016)
		Infrequent change of disinfecting solution	(Devonshire *et al*., 1993)*
		Matching of disinfecting solution and lens cases	(Wu *et al*., 2015)
Others	**Hand hygiene**	Hand washing	(Wu *et al*., 2015)
	Occupation	Type of work (clerical/non-clerical)	(Jiang *et al*., 2014)*
	Other behavioural factors	Exchange of the contact lenses	(Wu *et al*., 2015)
		Recommended contact lens cleaning protocol	(Wu *et al*., 2015)

*, represents the factors significantly ($p < 0.05$) associated with the level of lens case contamination.

1.7 CONTACT LENS DISINFECTING SYSTEM:

Contact lens care systems are an important part of the contact lens regimen. The goal of contact lens care systems is to minimise contact lens deposits and to combat microbial contamination. Contact lens disinfecting systems have included heat/thermal, chemical and ultraviolet disinfection (**Figure 1-1**) and in recent days the antimicrobial strategies have become an integral part of contact lens care system.

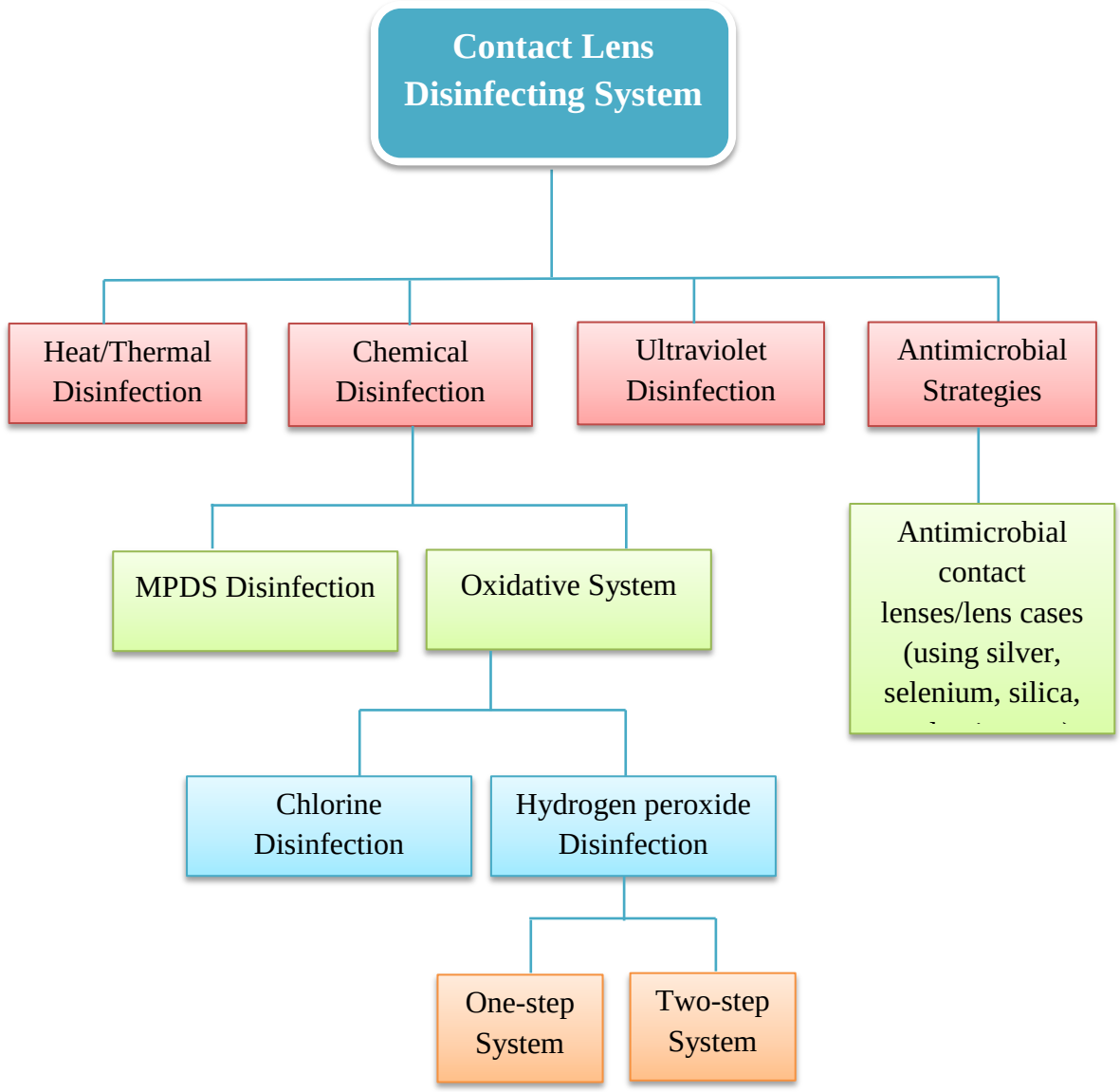

Figure 1-1: List of contact lens cleaning and disinfecting systems.

1.7.1 Thermal or Heat Disinfection

The heat systems were used in the 1970's but have fallen out of favour due to the denaturation of proteins adherent to the lenses (Garner, 1982). Whilst eradication of microbial contamination is best achieved by heat disinfection (Liubinas *et al*., 1987; Quesnel *et al*., 1996; Richardson *et al*., 1993), heat disinfection is not compatible with some contact lens materials with a high-water content because the required high temperature may cause molecular break down and dehydration of lenses (Rohrer *et al*., 1986).

1.7.2 Ultraviolet (UV) Light Contact Lens Disinfection System

Ultraviolet light has been used as one of the contact lens disinfection system. PuriLens was an UV light based cleaning and disinfection device of soft contact lenses and, by means of subsonic agitation, removed lens deposits and microorganisms (Bartolomei *et al*., 1994; Choate *et al*., 2000). Whilst UV radiation can kill microorganism, the direct exposure to UV radiation may cause damage to contact lenses. The storage solution of PuriLens disinfecting system was preservatives free (to avoid UV absorption) and did not contain chemicals that were effective against *Acanthamoeba* (Hwang *et al*., 2004).

1.7.3 Chlorine Systems:

Chlorine disinfecting systems were commonly used for oxidative chemical disinfection in the 1970s. Sauflon Pharmaceuticals developed an organic chlorine releasing system named SoftTab with the active ingredient of dichloroisocyanurate which demonstrated prolonged bactericidal effect with no major toxic reactions to the ocular surface (Copley, 1989). However, any increase in exposure time to chlorine-based disinfecting system may increase the margin of safety associated with the use of these products

(Penley *et al*., 1981). Also, the tinted lenses with reactive dyes can have their colour altered.

1.7.4 Hydrogen peroxide (H_2O_2) Disinfection System

The concept of using hydrogen peroxide system to disinfect contact lenses was introduced in the early 1970s (Aquavella *et al*., 1971). Hydrogen peroxide disinfecting systems are oxidative disinfection system which are formulated with 3% peroxide concentration at pH level of 3 - 4. To maintain the cellular structure and integrity of the corneal surface upon insertion of the lenses, a neutralisation step is required following disinfection. A low concentration of H_2O_2 is considered as a harmless chemical compound (usually within 100 ppm; 100ppm = 0.01%) to corneal cells because it breaks through hydrogen and water (Holden, 1990; Paugh *et al*., 1988). Depending on the neutralization step, H_2O_2 disinfecting system can be either a one-step or two-step system. The one-step process neutralizes lenses during the disinfecting, while the two-step process neutralizes lenses after the disinfecting step.

Hydrogen peroxide disinfecting system have activity both on the surface of lenses and it also penetrates the lens materials and cleans by expanding the lens matrix and oxidizing microbes (Gromacki, 2006). Hydrogen peroxide is a strong oxidising agent and acts on microbial cells in various ways including the denaturation of proteins, degeneration of lipids in bacterial cell walls and membranes, and alteration of DNA structure causing cell death (Finnegan *et al*., 2010). A one-step peroxide system (CLEAR CARE®, Alcon) has been shown to the most effective solution at reducing the bacterial contamination by clinical and reference strains of *Pseudomonas aeruginosa*, *Serratia marcescen*s and *Staphylococcus aureus* on contact lenses *in vitro,* compared to five different MPDS (Szczotka-Flynn *et al*., 2009).

Hydrogen peroxide disinfecting systems are also effective against fungal biofilms of *Fusarium oxysporum* and *Fusarium solani* when compared to other MPDS (Retuerto *et al*., 2012). The added advantage of using H_2O_2 disinfecting solution is the efficacy in killing *Acanthamoeba* cysts. The two-step H_2O_2 disinfecting solution has better efficacy against *Acanthamoeba* cysts compared to one-step solutions (Hiti *et al*., 2005). In one study, the use of one-step H_2O_2 disinfecting system was found more likely to be associated with microbial keratitis compared to the use of MPDS (Houang *et al*., 2001) however, the reverse was found in a recent study (Lim *et al*., 2016).

1.7.5 Multipurpose Disinfecting Solution:

Multipurpose solutions are combinations of different components that disinfect and remove contact lens deposits (Morgan & Efron, 2006; Woods & Morgan, 2004). Recent surveys have demonstrated that MPDS solutions are widely used by daily lens wearers, accounting for 90% of disinfecting solution used by daily soft lens wearers in the United Kingdom and Australia (Morgan & Efron, 2006; Woods & Morgan, 2004).

The two most common types of disinfectants in MPDS in the market are Polyquad (polyquaternium-1) and the polyaminopropyl biguanide (PHMB). All MPDS solutions pass through a regulatory process to ensure their safety and are required to exhibit a broad spectrum antimicrobial efficacy, based on the requirements of the International Organization for Standardization (ISO) 14729 stand-alone test (ISO, 2014). However, this procedure does not take into account the potential interactions between the biocide and contact lenses, lens cases, or contaminating organic materials (Eydelman *et al*, 2012).

Multipurpose disinfecting solutions have been modified on different occasions to improve comfort of lens wear and to reduce ocular signs and symptoms (corneal

staining, redness, burning sensation and itchiness) that can occur during lens wear. Cytotoxicity may vary between disinfecting solutions (Santodomingo-Rubido *et al*., 2006). However, the combination of MPDS with different contact lenses may still lead to toxic staining of cornea resulting that can lead to discontinuation of contact lens wear (Jones *et al*., 2002).

The performance of contact lens disinfecting solution varies based on the presence of biocide and the preservatives (Gabriel *et al*., 2018; Lin *et al*., 2016). Also, the presence of contact lenses can reduce the antimicrobial efficacy of MPDS specific to the biocide system and challenge organisms (Mohammadinia *et al*., 2012). For bacteria and against the species of *F. solani* and *C. albicans,* polyquaternium based solutions in combination with either Aldox or Alexidine demonstrated the most effective antimicrobial activity in presence or absence of lenses compared to solution containing PHMB (Gabriel *et al*., 2018; Stapleton *et al*., 2011; Willcox *et al*., 2010; Willson *et al*., 2014). The preservatives with the biguanide function group, chlorhexidine and polyaminopropylbiguanide (PAPB), had the best anti-staphylococcal activity, while EDTA was the best anti-pseudomonal preservative (Lin *et al*., 2016). The combination of chlorhexidine and EDTA had excellent synergy against *P. aeruginosa* (Lin *et al*., 2016).

Other dual disinfectants in MPDS (Kirk & Smick, 2011; Willcox, 2013) include polyquaternium-1 coupled with either alexidine or polyaminopropyl biguanide (**Table 1.7**). These dual disinfecting MPDS are especially effective against pathogens that are more difficult to kill, such as fungi and *Acanthamoeba* compared to the single disinfectant MPDS. However, despite the use of contact lens cleaning and disinfecting systems, microbial contamination of lens cases is high ranging between 30% to 85% in

asymptomatic wearers (Szczotka-Flynn *et al*., 2010; Willcox *et al*., 2010; Wu *et al*., 2015).

Table 1.7 Ingredients of dual disinfectants MPDS, adopted from (Willcox, 2013).

Product	Manufacturer	Disinfectants	Surfactants	Other ingredients
Old generation dual disinfecting solution				
Flexsol	Alcon Laboratories Inc.	Chlorhexidine and Thimerosal	-	-
Flex-Care	Alcon Laboratories Inc.	Chlorhexidine and thimerosal	-	-
New generation disinfecting solution				
Opti-Free® Express®	Alcon Laboratories Inc.	Polyquaternium-1 (Polyquad 0.001%); Aldox (myristamidopropyldimethylamine) 0.0005%,	Tectronic 1304	Ethylenediaminetriacetic acid; Sodium citrate; Sodium chloride; Boric acid; Sorbitol; aminomethylpropanol
Opti-Free® EverMoist®/ PureMoist®	Alcon Laboratories Inc.	Polyquaternium-1 (Polyquad 0.001%); Aldox (myristamidopropyldimethylamine)	Tectronic 1304	Ethylenediaminetriacetic acid; Sodium citrate; Sodium chloride; Boric acid; Sorbitol; Aminomethylpropanol
Opti-Free® RepleniSH®	Alcon Laboratories Inc.	Polyquaternium-1 (Polyquad 0.001%); Aldox (myristamidopropyldimethylamine) 0.0005%	Tectronic 1304	Nonanoyl-ethylenediamietriacetic acid, Sodium chloride, Sodium citrate, Sodium borate, Propylene glycol
RevitaLens OcuTec	Abbott Medical Optics.	Polyquaternium-1 (Polyquad 0.0003 %), Alexidine dihydrochloride (0.00016 %)	Tectronic 904	Ethylenediaminetriacetic acid; Boric acid; Sodium borate; Sodium citrate; Sodium chloride
Biotrue®	Bausch & Lomb	Polyaminopropyl biguanide (PAPB; 0.00013%), Polyquaternium-1 (Polyquad 0.0001%)	Poloxamine and Sulfobetaine	Ethylenediaminetriacetic acid; Hyaluronan; Boric acid; Sodium borate; Sodium chloride

1.7.6 New Antimicrobial Strategies

Different approaches have been introduced to reduce contact lens case contamination by developing the new storage case designs which can be easily cleaned to avoid biofilm formation (Caroline & Campbell, 1990; Kanpolat *et al*., 1992). Other strategies include attachment of materials on the lens case surfaces to discourage the adhesion of bacteria (Yung *et al*., 2007), incorporating disinfecting enhancing agents such as sodium salicylate in multipurpose disinfecting solutions which penetrates the biofilm (Farber *et al*., 1995), and the use of minimal concentration of macrolide antibiotics to inhibit the production of glycocalyx by sessile organisms (Dart, 1997; Donlan & Costerton, 2002; Parra-Ruiz *et al*., 2012). Attachment of antimicrobial compounds to contact lens case surfaces, is an alternative promising strategy (Amos & George, 2006; Cole *et al*., 2010; Dantam *et al*., 2012, 2011; Dutta *et al*., 2013; Qu *et al*., 2013). The antimicrobial coatings on lens cases can be silver, selenium, polyquads, polymeric pyredium compounds, nitric oxide, furanones or cationic peptides (Reid *et al*., 2013; Weisbarth *et al*., 2007). Each agent has a different mode of action on microorganisms to reduce adhesion or biofilm formation as shown in **Table 1.8**. Many microbial agents have more than one mechanism of actions to kill microbes.

Table 1.8 Antimicrobial agents and their mode of actions to inhibit microbial adhesion or biofilm formation.

Mode of actions	Antimicrobial agents
Deformation of the cell wall and membranes of microbes	Silver (Abu-youssef *et al*., 2010), Selenium (Michelle *et al*., 2016), Melimine (Rasul *et al*., 2010)
Inhibition of nucleic acid synthesis	Cationic peptides (Rasul *et al*., 2010), Silver (Dallas *et al*., 2011)
Antimetabolite activity	Cationic peptides (Rasul *et al*., 2010), Silver (Abu-youssef *et al*., 2010)
Interruption of bacterial respiration and synthesis of adenosine triphosphate	Silver (Batarseh, 2004; Kasuga *et al*., 2004; Klueh *et al*., 2000; Kumar & Münstedt, 2005; Shearer *et al*., 2000)
Inhibition of the production of teichoic acid (constituent of cell wall) and slime-associated protein	Sodium salicylate/ Salicylic acid (Muller *et al*., 1998), Sodium diclofenac and Ketorolac (Bandara *et al*., 2004; Hartog *et al*., 2010)
Inhibition of bacterial cell-to-cell signalling system (quorum sensing)	Fimbrolide (furanone) (Wu *et al*., 2015)
Suppression the adhesion of microorganisms	Phosphorylcholine (Selan *et al*., 2009)
Inhibition of protein synthesis	Selenium (Michelle *et al*., 2016), Sodium salicylate/ Salicylic acid (Muller *et al*., 1998), Sodium diclofenac and Ketorolac (Bandara *et al*., 2004; Hartog *et al*., 2010)

1.7.7 Antimicrobial Surface Coatings for Contact Lens Storage Cases

1.7.7.1 Silver coated antimicrobial contact lens storage case

Silver ions have been used to produce antimicrobial polymers in combination with several biomolecular components. Silver cations (Ag^+) are highly reactive and strongly bind to electron donor groups such as sulphur, oxygen, and nitrogen that are present in targeted microorganisms. Slow release of silver ions inhibits bacterial growth by multiple methods including enhancing structural deformities in nucleic acids (Dallas *et al*., 2011), cell membranes (Abu-youssef *et al*., 2010) and cell walls of bacteria (Abu-youssef *et al*., 2010; Cavicchioli *et al*., 2010; Dias *et al*., 2006; Ramstedt *et al*., 2007; Sambhy *et al*., 2006), and can interfere with bacterial respiration and synthesis of

adenosine triphosphate (Batarseh, 2004; Kasuga *et al*., 2004; Klueh *et al*., 2000; Kumar & Münstedt, 2005; Shearer *et al*., 2000). Silver nanoparticles have been used in contact lenses and contact lens storage cases to inhibit microbial contamination. Bacterial (and probably fungal) sensitivity to silver is genetically determined and relates to the levels of intracellular silver uptake and its ability to interact and irreversibly denature key enzyme systems (Dakal *et al.*, 2016; Lansdown, 2006, 2010). However, silver exhibits low toxicity in the human body, and minimal risk is expected due to clinical exposure by inhalation, ingestion, dermal application or through the urological or haematogenous route (Lansdown, 2006, 2010). Silver is absorbed into the human body and enters the systemic circulation as a protein complex to be eliminated by the liver and kidneys (Lansdown, 2006, 2010).

Silver is antimicrobial against the strains of *P. aeruginosa* and *S. aureus* (Nissen & Furkert, 2000). Later, Willcox *et al.* demonstrated the effect of silver nanoparticles to reduce microbial colonization on contact lens surfaces against the strains of bacteria and also against *Acanthamoeba* (Willcox *et al*., 2010).

Silver-impregnated lens cases show robust activity against both standard strains and clinical strains of Gram positive and Gram negative bacteria (Amos & George, 2006; Dantam *et al*., 2011). The multiple mechanisms by which silver ions kill bacteria do not allow bacterial cells to develop resistance which is an additional advantage over other disinfecting systems (Abu-youssef *et al*., 2010; Cavicchioli *et al*., 2010; Dallas *et al*., 2011; Dias *et al*., 2006; Ramstedt *et al*., 2007; Sambhy *et al*., 2006). However, the *in vivo* efficacy of silver-impregnated contact lens cases has shown mixed responses to reduce microbial contamination in normal lens wearers (Amos & George, 2006; Dantam *et al*., 2012). Commercially available silver coated antimicrobial silver lens

cases are Proguard (Ciba Vision Australia, GA), i-Clean (Sauflon Pharmaceuticals Ltd, London, UK) and Nano case (Marietta vision, Marietta GA). **Table 1.9** describes the antimicrobial efficacy of contact lens storage cases.

1.7.7.2 Silica nanoparticles

A robust surface-modified silica nanoparticles-based brush coating antimicrobial surface has been recently developed to resist protein and nucleic acid adsorption and prevent bacterial and cellular adhesion (Qu *et al*., 2013). Qu *et al*. developed brush coated surfaces of silica nanoparticles on polypropylene polymers of lens cases and demonstrated reduced bacterial adhesion forces of all challenged bacterial strains on brush coated silica surface compared to uncoated polypropylene cases (Qu *et al*., 2013), which may mean that the bacteria would then be easily removed from the surface of the lens cases during rubbing and rinsing of cases as is recommended as the hygiene procedure for cases (FDA, 2008).

1.7.7.3 Selenium

Organo-selenium has been attached to a variety of organic or polymer materials as a catalyst to produce antimicrobial surfaces (Spallholz *et al*., 2009; Tran & Webster, 2013). Selenium generates superoxide in the presence of oxygen and reduced thiol groups or other electron donating groups such as NADPH-dependent reductase or membrane proteins near target microorganisms (Spallholz, 1994). Selenium produces short lived antimicrobial superoxide (O_2^-) is non-toxic and non-carcinogenic to mammalian cells (Feigl & West, 1947; Palace *et al*., 2004). Mathews *et al*. showed in a pilot study that covalently bound organo-selenium compounds generate free radicals that inhibit bacterial proliferation and colonization in contact lenses and did not produce any contact lens related adverse ocular signs in rabbit models (Mathews *et al*., 2006). When selenium was covalently incorporated into the polypropylene polymer of

contact lens case showed bacterial biofilm inhibition of *S. aureus* (Reid *et al*., 2013). Organo-selenium has been incorporated onto different biomaterials such as haemodialysis catheters (Tran *et al*., 2012), and co-polymerized on tooth enamel to prevent dental plaque formation (Looney, 2009). The future of selenium coated contact lenses and cases looks promising. However, further optimization is required for selenium attachment to contact lens storage case and retention of the efficacy in human trials is yet to be demonstrated.

Table 1.9: Literature review on Antimicrobial contact lens storage cases and findings.

Author	Used contact lens storage cases	Antimicrobial agent	Method	*In Vitro/In Vivo*	Microorganisms assessed	Findings
(Amos & George, 2006)	Micro-Block™ (Ciba Vision Australia, GA)	Silver	Impregnation	*In vitro/ In vivo*	*Pseudomonas aeruginosa, Citrobacter amalonaticus, Serratia marcescens, Klebsiella pneumonia, Acinobactor calcoacetus, Escherichia coli*	Both *in vitro* and *in vivo* silver-impregnated cases reduced the number of recovered microorganisms compared to control cases. Case contamination rates were similar regardless of the contact lens storage case regimen followed.
(Dantam *et al*., 2011)	MicroBlock™ /Proguard (Ciba Vision Australia, GA), i-Clean (Sauflon Pharmaceuticals Ltd, London, UK), Nano case (Marietta vision, Marietta GA)	Silver	Impregnation	*In vitro*	Test organisms included ISO14729 Panel (ISO, 2014) and two clinical isolates, *Delftia acidovorans* and *Stenotrophomonas maltophilia*	Micro-Block cases were effective against most of the Gram negative bacteria. Only Micro-Block silver cases released measurable silver ions. i-Clean case was more effective against *S. aureus.*
(Wu *et al*., 2011a)	MicroBlock™ (Ciba Vision Australia, GA)	Silver	Impregnation	*In vitro*	*Pseudomonas aeruginosa Staphylococcus aureus*	Mechanical rubbing and wiping of silver lens cases reduced the amount of biofilm.

(Dantam *et al.*, 2012)	MicroBlock™ (Ciba Vision Australia, GA) Non-silver Regular lens case (Ciba Vision Australia, GA)	Silver	Impregnation	*In vivo*	Most commonly recovered microorganisms: *Propionibacterium* spp. *Staphylococcus epidermidis*, *Bacillus* spp. *Serratia liquefaciens*, Different fungi	Silver-impregnated cases were colonized by reduced level of Gram negative bacteria. Lower number of microbes recovered from silver cases when maintained wet.
(Qu *et al.*, 2013)	Non-adhesive Silica nano-particles based brush coated lens cases (Ispa Plastics, Groningen, The Netherlands)	Silica	Brush coated	*In vitro*	*Pseudomonas aeruginosa*, *Serratia marcescens*, *Serratia liquefaciens*, *Staphylococcus aureus*	All challenged bacteria were recovered in significantly less number on brush coated than uncoated polypropylene cases.
(Reid *et al.*, 2013)	Selenium coating on the polymer of contact lens case material	Selenium	Covalently incorporated	*In vitro*	*Staphylococcus aureus*, *Stenotrophomonas maltophilia*, Different fungi	Selenium containing polypropylene showed over 7 logs (complete) inhibition against challenged microorganisms.

1.8 BIOFILM FORMATION IN CONTACT LENS CASE

The use of commercially available cleaning and disinfecting systems along with recommended hygiene instructions might not be effective at keeping lens cases free of contamination (Stapleton *et al*., 1995; Wu *et al*., 2010b). Microbes are more often found in association with multispecies biofilm formation compared to contact lenses and other contact lens related accessories (McLaughlin-Borlace *et al*., 1998). Bacteria in contact lens cases may form biofilms through the process of adhesion, coaggregation and cohesion. *In vitro* and *in vivo* studies, including the use of confocal microscopy, have detected bacterial biofilm in contact lens storage cases (Farber *et al*., 1995; Mckenney & Ajello, 1991; McLaughlin-Borlace *et al*., 1998), but no mechanistic studies have been published.

1.8.1 Mechanisms of Bacterial Biofilm Formation

Biofilm is defined as an assemblage of microbial cells irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Costerton *et al*., 1995). Bacterial biofilm formation generally develops through similar mechanisms irrespective of the ecosystem (Donlan & Costerton, 2002; Kroos & Kaiser, 1987; Rickard *et al*., 2003) and is governed by physical, chemical and biological processes (Dang & Lovell, 2000; Marsh, 2004). The following four specific steps are involved in biofilm formation: (a) attachment of cells to a substrate (adhesion) followed by (b) cell-to-cell attachment (coaggregation) (Chen & Wen, 2011; Kolenbrander, 2000), (c) development of a mature biofilm and (d) eventually cell dispersion from the biofilm. Coaggregation between single species or multiple species can occur during the process of biofilm formation (Kolenbrander *et al*., 2002). Cohesion can also occur, which is described as the ability of a pioneer species of microbe to promote adhesion of

subsequent species (**Figure 1-2**). Coaggregation is mainly controlled by surface adhesins and cohesion can be controlled by cell-to-cell communication signalling pathways, depending on the types of bacterial species (Kaiser & Losickt, 1993; Kroos & Kaiser, 1987).

1.8.1.1 Adhesion and biofilm formation:

Initially, planktonic bacteria move to a material's surface by Brownian motion, Van-Der-Walls attraction forces and gravitational forces, then slowly adhere to the surface (Krekeler, Ziehr, & Klein, 1989). Absorbed cells become irreversibly adhered to the substrate (Rutter & Vincent, 1980). Factors including surface energy, surface functionality, bacterial orientation, temperature and pressure contribute to this adhesion stage (Garrett *et al*., 2008; Rickard *et al*., 2003). A conditioning film can form in a contact lens case when a lens case is in contact with MDPSs. This conditioning film can be composed of organic molecules some of which may also be derived from tears and skin (Bruinsma, Mei, & Busscher, 2001). The conditioning film influences the adherence of microbes (Bruinsma *et al*., 2001; Willcox *et al*., 2001).

1.8.1.2 Cohesion and coaggregation and their role in biofilm formation:

Adherent bacteria may encourage other bacteria to adhere to the primary colonizers or the substratum and this helps to consolidate biofilm formation. This can occur by pioneer microbes providing new surfaces (their own surface) for other bacteria to adhere to (i.e. via coaggregation) or by the pioneer microbes modifying the surface itself to facilitate adhesion of secondary colonisers (**Figure 1-2**). Carbohydrates of *Streptococcus mutans* adhere to the tooth surface and promote more bacteria to adhere to the surface (Cisar *et al*., 1979; Sato *et al*., 1984). Additionally, extracellular polysaccharides can mediate cohesion between certain strains of *Streptococci mutans*, *Streptococcus oralis* and *Neisseria pharynges* (Willcox *et al*., 1990).

Coaggregation is the specific recognition and adherence of bacteria to themselves or other microbial types and can be associated with the development of complex multispecies biofilm (Cisar *et al*., 1979; Gibbson & Nygaard, 1970; Mcintire *et al*., 1978). Bacterial coaggregation was first observed in bacteria isolated from dental plaque (Kolenbrander, 2000) and later in the mammalian gut, human urogenital tract and in the portable water supply systems (Rickard *et al*., 2002).

S*treptococci* are commonly found first colonizers of the tooth surface and coaggregated with Gram positive rods such as *Actinomyces naeslundii* (Kolenbrander, 1991) builds up the biofilm (plaque). This results in a nutritionally beneficial, mutualistic relationship that enables each bacterial species to grow (Nyvad & Kilian, 1990).

Different mechanisms are involved in coaggregation depending on the types of bacterial species involved. Coaggregation adhesins are usually found on the cell walls of micro-organisms and mediate binding to cognate receptors on the reciprocal organisms. Coaggregation is often mediated by protein–saccharide interactions and can be blocked by the addition of simple sugars. Coaggregation adhesins have been identified on *Actinomyces, Streptococcus* and *Fusobacterium* species (Kolenbrander, 1991; Kolenbrander & Williams, 1981; Nyvad & Kilian, 1990) amongst other bacterial types.

Coaggregation adhesins can be located away from the bacterial cell wall on external appendages of bacterial cells thus enabling cells to make more effective contact with prospective partners (Busscher *et al*., 1992; Sandberg *et al*., 1995). Microorganisms can express more than one coaggregation adhesin simultaneously on the cell surface;

this will also optimize the chances of a cell finding a suitable partner in the competition for survival in the high-shear oral environment (Rickard *et al*., 2002).

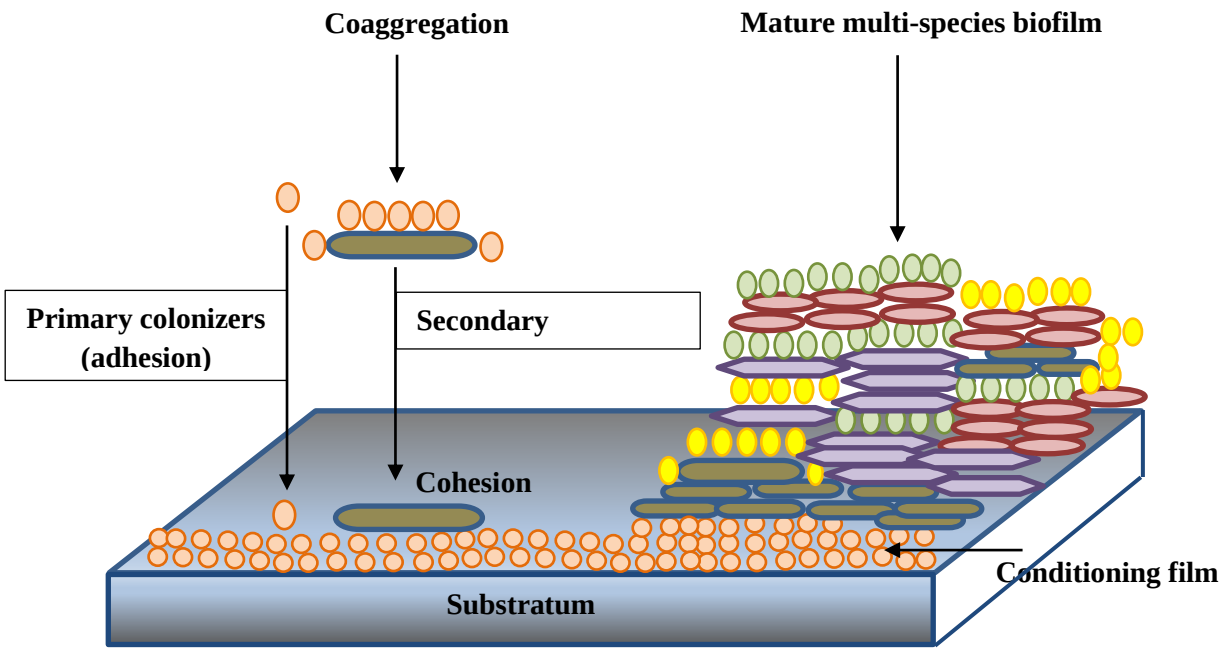

Figure 1-2: Bacterial coaggregation in dental plaque formation.

Coaggregation inhibition:

The nature of biochemical interactions during coaggregation has been explored using various coaggregation inhibitory substances. Lactose, galactose, glucose, sucrose and N-acetyl-D-galactosamine have been used as inhibitory substances **Figure 1-3**. Coaggregation inhibition by lactose between *Actinomyces viscosus* 19246 and *Streptococcus sanguis* 10557 confirmed the involvement of a proteinaceous substance on the cell surface of the *Actinomyces* with a carbohydrate site on the streptococcus cells (Sato *et al*., 1984).

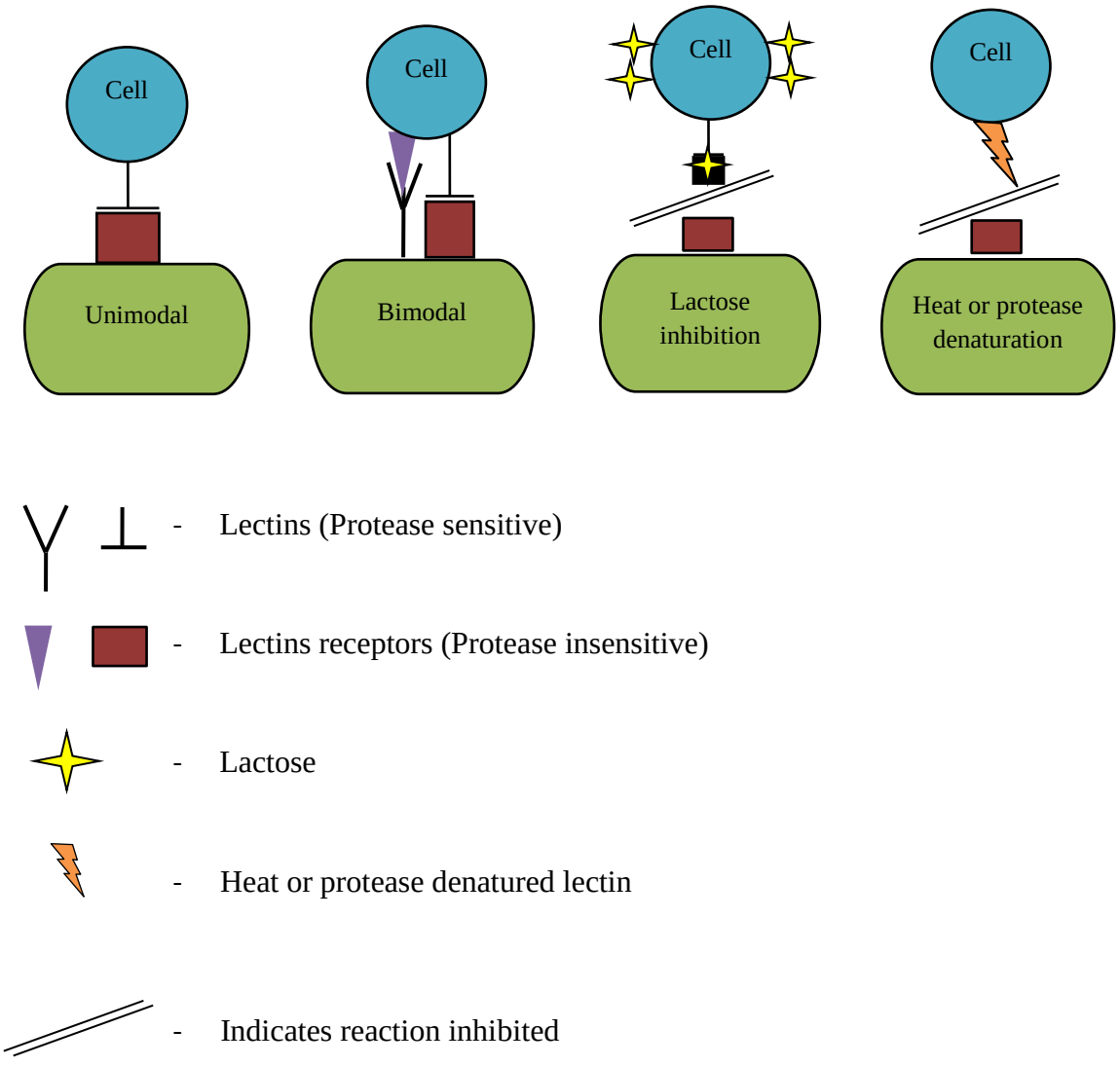

Figure 1-3: Interactions between coaggregating pairs of organisms.

1.8.1.3 Maturation and cell dispersion:

Biofilm maturation often ends with cell dispersion characterized with the shedding of daughter cells from the actively growing bacteria (Donlan, 2002). A number of proteins and exopolymeric substances which cause oxidative stress and eventual cell dispersion can be differentially produced during the various stages of biofilm development (Sauer, 2003). Overproduction of these proteins and exopolymeric substances, often accompanied by the nutrient depletion, causes cell dispersion and reversion of cells into a planktonic state (Oosthuizen *et al*., 2002; Sauer *et al*., 2002). Alginate lyase produced by *P. aeruginosa*, N-acetyl-heparosan lyase produced by *Escherichia coli* and hyaluronidase produced by streptococci are the examples of enzymes produced by mature bacterial cells which can cause the dispersion of mature biofilms (Sutherland, 1999).

1.8.2 Bacterial growth inhibition during biofilm formation:

During biofilm formation certain bacteria release a variety of virulence factors including exotoxins, pyocyanin, proteases, hemolysins, and QS molecules to infect host cells or outcompete other microorganisms for nutrients in mixed microbial communities (Byng *et al*., 1979; Kim *et al*., 2015a; Schuster & Greenberg, 2006).

Bacterial growth inhibition between the species of *Staphylococcus* spp. and *P. aeruginosa* has been well documented (Atalla *et al*., 2011; Biswas *et al*., 2009; DeLeon *et al*., 2014; Hoffman *et al*., 2006; Proctor *et al*., 2006). For example, in the ecological niche of wound and lung infections, the relationship between *P. aeruginosa* and *S. aureus* is competitive rather than cooperative where *P. aeruginosa* releases pseudomonas quinolone signal and secretes toxic substances, such as alkyl-hydroxyquinoline N-oxides, hydrogen cyanide, and pyocyanin, that impede the proliferation of *S. aureus* (Biswas *et al*., 2009; DeLeon *et*

al., 2014). Additionally, the production of LasA endopeptidase by *P. aeruginosa* induces lysis of *S. aureus* (Biswas *et al*., 2009; DeLeon *et al*., 2014). However, *P. aeruginosa* does not completely stop the growth of *S. aureus*, partly because *S. aureus* has also devised strategies (Atalla *et al*., 2011; Biswas *et al*., 2009; Hoffman *et al*., 2006; Proctor *et al*., 2006) to survive in the presence of *P. aeruginosa* such as electron transport-deficient small-colony variants, staphyloxanthin and staphylococcin (Biswas et al., 2009; Nair, Biswas, Götz, & Biswas, 2014).

P. aeruginosa and *S. aureus* often have been cultured together from the contact lens cases of individuals with contact lens induced microbial keratitis (Mayo *et al*., 1987; McLaughlin-Borlace *et al*., 1998; Stapleton *et al*., 1995). However, there is limited evidence on the mechanisms underlying co-existence of *P. aeruginosa* and *S. aureus* among the strains of ocular surface and contact lens cases which may have a large impact on the clinical outcome of a patient and therefore should be a subject of continuing investigation.

1.9 RESEARCH GAPS:

Overall, bacterial biofilm formation is complex comprising several stages, starting with adhesion, micro-colony formation through the process of cohesion, coaggregation, and eventual formation of a mature biofilm (Rickard *et al*., 2003). From the previous discussion it has been demonstrated that bacterial biofilm formation is well established in the oral ecosystem (Marsh, 2004). It has been assumed that bacteria follow a similar process in biofilm formation irrespective of the ecosystem.

Contact lens cases often become contaminated with multiple bacterial species, analogously to dental plaque formation in the oral ecosystem. Specific contact lens case isolates are known to be biofilm producers, however, the mechanism of biofilm formation within a multispecies environment is yet to be elucidated. The reported rates of lens case contamination, and the types of bacterial species isolated from lens cases vary in different studies. These data highlight the fact that there is very little known about bacterial interactions in cohesion, coaggregation and mature biofilm during contact lens case contamination. Thus, it is important to understand the interactions that facilitate the bacterial colonization in lens cases of the most commonly isolated bacteria from lens cases.

Contaminated storage cases might serve as initial source for the transmission of microbes to contact lenses and eventually to ocular surface (Vermeltfoort *et al*., 2008). Non-compliance behavior in contact lens wear may contribute to microbial contamination of lens cases (Wu *et al*., 2015). To address this issue, different strategies have been adopted and introduced in the market such as new contact lens cleaning and disinfecting solution and antimicrobial storage cases. However, the information about the antimicrobial efficacy of these new disinfecting systems are sparse. Therefore, it is

relevant to evaluate the performance of these novel products to reduce the storage case contamination and eventually it is likely to reduce microbially driven adverse events and promote safe contact lens wear.

1.10 RATIONALE FOR RESEARCH

Contact lens cases are often contaminated during use of daily wear lenses. Microbial contamination of contact lens cases is found in up to 85% of asymptomatic wearers, despite using contact lens disinfecting solutions. Lens case contamination has also been linked to the development of contact lens induced microbial keratitis. Contact lens induced corneal infection is still a major barrier in the growth of contact lens industry.

Therefore, the rationale behind this study is to estimate the rate of contact lens case contamination using the povidone-iodine based disinfecting system and to identify the types of bacterial species that colonize lens cases. Subsequent experiments are designed to understand the interaction that may facilitate the bacterial colonization of the most commonly isolated bacteria from lens cases. Especially, these studies have aimed to examine the pattern of adhesion, coaggregation, cohesion and production inhibitor substances. The study findings may help to identify better approaches to prevent bacterial contamination by targeting specific bacteria which accelerate the process of bacterial colonization.

Additionally, to examine *in vitro* and *in vivo* colonization of antimicrobial silver and non-silver contact lens cases. The present study also has investigated whether antimicrobial contact lens storage case can reduce the rate of bacterial colonization and changes the types of microbes that can be cultured from lens cases during use. The

study outcome may lead to new approaches to eliminate contact lens case contamination, thus minimizing contact lens associated ocular complications.

1.11 THESIS AIMS AND HYPOTHESIS

Aims:

- To investigate the rate of contact lens case contamination and the types of microorganisms recovered from lens cases with a new disinfecting system.
- To investigate whether bacterial coaggregation or cohesion facilitates or production of bacterially produced inhibitory substances modulates bacterial colonisation on contact lens cases.
- To evaluate the biocidal efficacy of silver copolymerized barrel lens cases in laboratory-based experiments.
- To evaluate the rate, level and types of microbial contamination to antimicrobial contact lens cases, *in vivo.*
- To understand the association between the compliance of lens wear and the level of microbial contamination to antimicrobial lens cases, *in vivo*.

Hypotheses:

- Povidone-iodine based disinfecting solution is effective in reducing microbial contamination.
- An organised mechanism is involved in the bacterial colonisation in lens cases.
- Silver antimicrobial lens cases are effective in reducing bacterial contamination.

1.12 THESIS OVERVIEW

Chapter 2 investigates the rate of contact lens storage case contamination and the types of microbial species isolated from contact lens storage cases after using a novel Povidone-iodine disinfecting system in a prospective *in vivo* trial.

Chapter 3 & 4 investigates and quantifies the bacterial coaggregation and cohesion and growth between the most commonly isolated bacterial species from contact lens storage cases.

Chapter 5 reports on the anti-microbial efficacy of the silver-impregnated storage cases against the planktonic and adhered bacteria, through the development of a repeatable method, by adapting a modified version of the ISO 14729. Also, it provides the further insight of the antimicrobial efficacy of silver lens storage case in conjunction with contact lens cleaning and disinfecting solution, contact lens and organic soil.

Chapter 6 investigates the clinical performance of new antimicrobial silver-impregnated contact lens storage cases in a prospective, cross-over, randomised, appropriately powered, double-masked, clinical trial.

Chapter 7 summarises the findings from the experimental Chapters 2 to 5 and repots the implication of the work described in this thesis, states the limitations of this thesis and proposes further studies.

Chapter 2:
Contact lens case contamination using a povidone-iodine disinfection system.

This chapter has been published as:

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  (Contribution: data collection, laboratory based microbial culture collection and identification, statistical analysis and manuscript preparation)

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OPHTECS Corporation provided the cleadew contact lens disinfecting solution for the clinical trial, and sponsored the running of the trial.

Chapter 2 Contact lens case contamination using a povidone-iodine disinfection system

2.1 INTRODUCTION

Almost a decade ago, global estimates indicated that there were more than 140 million contact lens wearers worldwide (Nichols, 2015). Discontinuations from contact lens wear due to discomfort and dryness remain, even with contemporary lens types (Dumbleton *et al*., 2017), and this has been identified as a factor hampering growth of the contact lens market (Pritchard *et al*., 1999). Nevertheless, the overall number of contact lens wearers is expected to rise given the growth in use of contact lenses to control myopia progression in children, and the uptake of multifocal contact lens corrections as the population ages (Efron *et al*., 2010; Efron *et al*., 2015).

Contact lenses are generally considered to be a safe form of vision correction. However, adverse events do occur. Acute infectious and inflammatory/infiltrative complications associated with contact lens wear present a considerable health and economic burden to both affected individuals and to public health systems. In addition to the severe pain and potential for loss of vision with microbial keratitis, the median treatment cost for microbial keratitis in Australia and New Zealand has been estimated at over AU$1200, which includes both the direct cost of health care, plus indirect costs related to time off work and costs associated with assistance from a care-giver (Keay *et al*., 2006). While corneal infiltrative events are less severe and typically associated with discomfort and inconvenience caused by discontinuation of contact lens wear (Carnt *et al*., 2009), the cost per contact lens-associated corneal infiltrative event is still high, ranging from US$1003 to 1496 (Smith *et al*., 2017). Therefore, eliminating or

minimizing the risk factors for developing lens-related complications is desirable for both affected individuals and society in general.

Microbial contamination of contact lens storage cases, used during storage of lenses when not being worn, is an important consideration given the potential for pathogenic micro-organisms in the lens case to colonize contact lenses and be transmitted to the eye (Qu *et al*., 2011). Microbial colonization of contact lenses has been implicated in contact lens-induced corneal inflammatory events (Sankaridurg *et al*., 2000; Szczotka-Flynn *et al*., 2010; Willcox *et al*., 2011). Contact lens case contamination rates range from 18% to 85% (Willcox, 2013).

In a study examining the rates of non-infectious keratitis during wear of silicone hydrogel contact lenses, Carnt *et al*. found that the rate was dependent on the contact lens-multipurpose disinfecting solution combination, with the lowest levels of corneal infiltrative events being produced when silicone hydrogel contact lenses were used in combination with a one-step hydrogen peroxide disinfecting system (Carnt *et al*., 2009). Analysis of the contact lens cases used during the clinical trials reported by Carnt *et al*. (Carnt *et al*., 2009) demonstrated that lens cases were contaminated during use (Willcox *et al*., 2010), and a further analysis showed that the rate of corneal infiltrative events was correlated to the level of microbial contamination of lens cases (Willcox, 2013).

Povidone-iodine (PVP-I, a commonly used medical disinfectant) has been available as a contact lens care solution in Japan for a number of years (Kilvington, 2004; Martín-Navarro *et al*., 2010), however no studies have reported the rates of adverse events in contact lens wearers using povidone-iodine, nor the rate of contamination of contact lens cases with this solution. Studies have demonstrated excellent *in vitro*

antimicrobial activity of 5% povidone-iodine against a number of clinical strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* (including methicillin resistant strains), *Candida albicans* and *Acanthamoeba* (Demirbilek & Evren, 2014; Gatti *et al*., 1998; Manuj *et al*., 2006; Yanai *et al*., 2006). Versions of a povidone-iodine cleaning and disinfecting system have been also shown to be effective against *Acanthamoeba* and *Fusarium* when tested *in vitro* (Kilvington *et al*., 2013; Kobayashi *et al*., 2011; Martín-Navarro *et al*., 2010). The aim of this study was to assess the rate of adverse events during wear of contact lenses and use of a povidone-iodine disinfecting solution (cleadew™ cleaning and disinfecting system, Ophtecs Corp, Kobe, Japan), and the rate and level of contamination in contact lens cases.

2.2 MATERIALS AND METHODS

2.2.1 Study design

This was a prospective, single centre, open label, controlled study evaluating the use of cleadew™ (**Figure 2-1**) cleaning and disinfecting system in existing, frequent replacement daily wear soft hydrogel and silicone hydrogel contact lens wearers over a three months period. Details of the cleadew™ cleaning and disinfecting system are shown in **Table 2.1**.

Table 2.1: Cleadew™ cleaning and disinfecting system (adopted from "Cleadew™ Investigator's Brochure and safety Evaluation manual", OPHTECS Corporation).

		First Care	First Care CT	Clencide
Disinfecting, neutralizing and cleaning tablet (220~240mg/ tablet)	Disinfecting	PVP-I (4mg/tablet)		
	Neutralizing	Ascorbic acid (2.0mg/tablet)	Sodium sulfite (2.4mg/tablet)	Sodium sulfite (2.4mg/tablet)
	Cleaning	Protease (0.5mg/tablet)	Protease (3.2mg/tablet)	Protease (8.0/tablet)
Dissolving and rinsing solution		Boric acid(1.5mg/mL), Sodium borate (0.16mg/mL), Sodium chloride (5.3mg/mL), hydroxy-ethane-tetrasodium-liquiddiphosphonic acid (0.3mg/mL), hydrogen peroxide (40ppm)	Boric acid(1.5mg/mL), Sodium borate (0.16mg/mL), Sodium chloride (5.3mg/mL), Disodium edetate (0.2mg/mL)	

Address: 5-2-4 Minatojimaminamimachi, Chuoku, Kobe, 650-0047, Japan

Figure 2-1: Cleadew™ povidone-iodine based cleaning and disinfecting solution. (Source of picture: Manual of Cleadew™, OPHTECS Corporation).

2.2.2 Ethics approval

This study was approved by the University of New South Wales (UNSW) Human Research Ethics Committee (approval ref # HC14270) and all procedures were conducted in accordance with the tenets of the Declarations of Helsinki 1975 as amended in 2000 including local regulations as applicable such as Therapeutic Goods Administration, Australia (TGA). The clinical trial was conducted under the clinical trial notification (CTN) scheme following the regulations of the Therapeutic Goods (Medical Devices) Regulations 2000.

2.2.2.1 Sample size calculation

Assuming a lens case contamination rate of 80% from previous studies and the expectation that povidone-iodine based disinfecting solution will demonstrate a significant reduction of 50% (80% *vs* 40%) in case contamination. This sample was estimated at 5% level of significance with 80% power and assuming a 20% drop out rate. Sample size calculation was conducted using the online sample size calculator tool as per http://stat.ubc.ca/~rollin/stats/ssize/index.html.

2.2.2.2 Key inclusion criteria:

- Be able to read and comprehend English and give informed consent as demonstrated by signing a Participant Information Statement and Consent Form (Appendix B1);
- Be at least 18 years old;
- Have ocular health findings which would not prevent the participant from safely wearing contact lenses.
- Be existing frequent replacement contact lens users (hydrogel or silicone hydrogel) who are willing to wear contact lenses on a daily basis for a minimum of 4 days per week (on average), over the course of the study;
- Be willing to use the study prescribed contact lens cleaning and disinfecting solution for the duration of the study;
- Be willing to return the used study contact lens cases and comply with the lens wear and study visit schedule as directed by the investigator;

2.2.2.3 Key exclusion criteria:

Participants enrolled in the trial must NOT:

- Use daily disposable contact lenses or be a rigid gas permeable lens wearer (including orthokeratology);

- Have history of skin allergy towards any metal or chemical components in particularly with iodine sensitivity were excluded from the study enrolment;
- Have any active corneal infection (by physical investigation), past ocular disease or systemic disease (by history taking) such as diabetes, Graves disease, and auto-immune diseases such as ankylosing spondylitis, multiple sclerosis, Sjögren syndrome and systemic lupus erythematosus that would affect wearing of contact lenses or may interfere with the ocular surface properties, were excluded from prospective study participation;
- Have use or need for any systemic or topical medications which may alter normal ocular findings/are known to affect a participant's ocular health/physiology or contact lens performance either in an adverse manner or risk providing a false positive;
- Have undergone eye surgery within 12 weeks immediately prior to enrolment for this trial;
- Have any contraindications to contact lens wear;
- Be pregnant at the time of enrolment in the clinical trial (self-reported questionnaire);
- Be currently enrolled in another clinical trial.

2.2.2.4 Safety reporting

A data safety monitoring board (DSMB) of independent individuals, external to the trial who are experts in relevant areas, was formed with the approval from Human Research Ethics Committee, UNSW to report any unexpected adverse event during the clinical trial.

2.2.3 Participant's enrolment and study instructions:

Forty (40) participants who gave written informed consent were dispensed with cleadew™ cleaning and disinfecting solution for use with a fresh (new) pair of their regular soft contact lenses. Participants were instructed to wear their contact lenses for a minimum of four days per week on average for the duration of the study, and to replace the lenses according to the manufacturers recommended schedule. Upon daily removal of the lens from the eye, participants were advised to place lenses directly into the appropriate case compartment and close the basket. The lens case was to be filled to the indicated line with cleadew™ dissolving and rinsing solution and a cleadew™ tablet added to the lens case. The lens case was to be capped and lenses left in the case for a minimum of four hours (the manufacturer's recommended minimum disinfection time). Upon removal of the contact lenses from the case, participants were instructed to rinse their lenses with the cleadew™ dissolving and rinsing solution prior to inserting lenses into their eyes. Solutions in the lens case were to be discarded and the lens case (lens well and baskets) was to be thoroughly rinsed with the cleadew™ dissolving and rinsing solution and left to air dry face down on a clean tissue when not in use. Participants were instructed to use the lens cases for 1 month, to return the lens cases to the clinic at the scheduled one month and three-month visits and use a new lens case each month. Participants were provided with a $20 voucher per visit, to thank them for their time.

2.2.4 Clinical examinations:

Clinical examinations were conducted at the baseline, one month and three month scheduled visits. At each visit, compliance with the minimum lens wear requirements and solution regimen were verified and subjective symptoms and feedback were obtained via a self-administered visual analogue scale questionnaire. Anterior ocular

health, including bulbar and limbal conjunctival redness, extent of corneal and conjunctival staining, and palpebral redness and roughness were graded using the CCLRU grading scales (Appendix E) (Terry, Cristina, Holden, & Cornish, 1993) using a Zeiss SL-120 biomicroscope (Carl Zeiss Meditech, Jena, Germany). The subjective ratings and ocular health variables measured at baseline were considered to represent the clinical performance of the participant's habitual lens care product, whereas assessments conducted at one month and three months were representative of the cleadew™ cleaning and disinfecting system with the participant's habitual contact lenses. The clinical trial was conducted under a similar protocol to that reported previously (Carnt *et al.*, 2009) with the exception that the participants used their habitual contact lenses rather than being supplied with a particular lens type.

2.2.5 Contact lens case analysis

Lens cases were collected at the one and three month visits. The lens cases were transferred to the laboratory within one hour of collection for analysis. Microbial analysis of the collected cases followed the same protocol as described previously (Willcox *et al.*, 2010). For contact lens case sampling, swabs were taken from the inside of the contact lens case (the case well, lid and basket) and suspended in sterile phosphate buffered saline (PBS). After mixing thoroughly, 400 µL aliquots of the saline were plated onto three chocolate agar plates (Thermo Fisher Scientific, Australia) and one Sabouraud's agar plate (Thermo Fisher Scientific, Australia). The chocolate agar plates were incubated either aerobically, anaerobically or micro-aerophilically at 37ºC for 48 hours, and then the colonies examined. Colony morphology was recorded, and each type of morphology was subjected to a Gram stain. The Sabouraud's agar plates were incubated at 25ºC for seven days to culture for

yeast and moulds and the colony morphology recorded. The number of colony forming units was recorded for each colony type. For eleven participants, separate swabs were used for the different case compartments to determine whether there were differences between these sites in contamination rates or types of microbes.

2.2.6 Bacterial identification protocol:

Identification of the types of bacteria contaminating lens cases was performed using 16S rRNA sequencing. In brief, DNA of each sample was extracted (using a QIAamp DNA Minikit; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Following extraction, amplification of the 16S rRNA sequence was performed in a total volume of 25 µL containing 1 µL of DNA template, 12.5 µL of EconoTaq Plus 2x Master Mix (Lucigen, Middleton, USA), 10.5 µL of DNAse free water and 1 µL each of 10 µM universal forward primer (F27 5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (R1492; 5'-CGG TTA CCT TGTTACGACTT-3'). Genomic DNA of *Escherichia coli* and DNAse free water were used as the positive and negative controls respectively. The polymerase chain reaction (PCR) was performed in a thermal cycler (Bio-Rad, Hercules, CA, USA) at the following settings: initial denaturing at 94ºC for 5 min followed by 25 cycles of denaturing at 94ºC for 30 seconds, annealing for 30 seconds at 56ºC, extension at 72ºC for 90 seconds, and a final extension step at 72ºC for 10 minutes. Electrophoresis of the amplified products was performed in a 1% agarose gel stained with 30 ppm of GelRed^{TM} 10000x solution in DMSO (Biotium, Hayward, CA, USA) to ensure single bands were obtained. The PCR products were then cleaned using Sephadex G-50 (GE Lifescience, Uppsala, Sweden) columns and sequenced using forward and reverse primers separately with the BigDye Terminator v3.1 (Applied Biosystems, Austin, TX,

USA). PCR amplification was performed using the following protocol: 99 cycles of PCR reaction run at the following conditions: 1 mins for 96ºC, 10 secs for 96ºC, 5 secs for 50ºC and 4 mins for 60ºC. Sample maintained at 4ºC and then stored at 4ºC. Post PCR products were again cleaned up by using Safodex G-50 as described earlier.

The sequenced samples were purified using Sephadex G-50 columns and analysed in an Applied Biosystems 3730 DNA Analyzer at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, NSW, Australia). The sequences were manually trimmed using Sequence Scanner v1.0 software (Applied Biosystems) and the forward and the reverse sequences assembled using DNA Baser v3.5.0 (Heracle BioSoft, SRL Romania). The Basic Local Alignment Search Tool (BLAST) of the National Centre of Biotechnology Information (www.ncbi.nlmnih.gov) database was used to identify the aligned sequences.

2.2.7 Statistical analysis of data

One-way analysis of variance (ANOVA) was used to compare data across the three visits, and the level of significance was set at alpha = 0.05. Bonferroni correction was used, and adjustments made for multiple comparisons where applicable. Overall contamination rates and contamination rates for different types of micro-organism were measured and tabulated. Also, repeated-measures ANOVA was used to compare the ocular signs of subjects and the data of the compliance questionnaire to determine any association with microbial contamination between visits.

2.3 RESULTS

2.3.1 Clinical performance of the povidone-iodine solution

A total of 40 participants (11 males and 29 females) with an average age of 30 ± 13 years (range 18 to 60 years inclusive) were enrolled and completed the study. However, two participants who presented to the three-month visit reported not having worn their contact lenses for 1 week prior to the final visit. Therefore, the three-month data for these subjects were excluded from the analysis. Thirty-four participants wore silicone hydrogel contact lenses and the most common lens worn was senofilcon A (by 17 participants) followed by comfilcon A (by eight participants). Four participants routinely used a peroxide disinfection system prior to the study, while the remainder used a variety of multipurpose solutions. **Table 2.2** describes the lens/solution combinations used habitually by the participants before enrolment in the study.

Ocular variables were measured for both eyes, but as there were no differences between the eyes upon statistical analysis only data for the left eye have been presented (**Table 2.3**). No significant differences were found between baseline compared to the one month or three-month visits for bulbar and limbal conjunctival redness, extent of conjunctival staining and palpebral redness and roughness ($p > 0.05$). However, the extent of corneal staining was significantly lower when cleadew™ was used at the three-month visit compared to participants' habitual lens care product at baseline ($p < 0.01$).

Table 2.2: Habitual lens and disinfecting solutions used by the participants, before enrolment in the study.

Contact lens Material (Manufacture)	No. of subjects	Disinfecting solution	No. of subjects
Silicone hydrogels		AOSept (hydrogen peroxide; CIBA Vision)	3
Senofilcon A (Johnson & Johnson Vision Care, Inc., Jacksonville, FL)	17	ReNu® Easysept (hydrogen peroxide; Bausch & Lomb, Rochester, NY)	1
Comfilcon A (Cooper Vision, Pleasanton, CA)	8	AQuify® (polyhexanide; CIBA Vision)	1
Lotrafilcon B (Alcon Laboratories Inc.)	5	ReNu® (polyaminopropyl biguanide; Bausch & Lomb)	9
Enfilcon A (Cooper Vision, Pleasanton, CA)	1	Biotrue® (polyaminopropyl biguanide and polyquaternium; Bausch & Lomb)	3
Balafilcon A (Bausch & Lomb)	1	Complete (polyhexamethylene biguanide; Abbott Medical Optics, Santa Ana, CA)	2
Galyfilcon A (Johnson & Johnson Vision Care, Inc.)	1	OPTI-FREE® Puremoist® (polyquaternium-1 and myristamidopropyl dimethylamine; Alcon Laboratories Inc.)	10
Hydrogels		OPTI-FREE® RepleniSH® (polyquaternium-1 and myristamidopropyl dimethylamine; Alcon Laboratories Inc.)	7
Etafilcon A (Johnson & Johnson Vision Care, Inc.)	1		
		OPTI-FREE® Express® (polyquaternium-1 and myristamidopropyl dimethylamine; Alcon Laboratories Inc.)	1
Ocufilcon D (Cooper Vision, Pleasanton, CA)	1	Unknown	3
Methafilcon A (Cooper Vision, Pleasanton, CA)	1		
Hilafilcon A (Bausch & Lomb)	1		
Other hydrogels (overseas/non-English label brands)	3		

Table 2.3: Objective and subjective variables recorded at each visit to the clinic.

	Baseline (n=40)	**1 month (n=40)**	**3 months (n=38)**	**ANOVA**
	Clinical variable (scale = 0-4)			
Bulbar redness – Nasal	1.9 ± 0.4	1.9 ± 0.4	1.7 ± 0.4	*0.06*
Bulbar redness – Temporal	1.9 ± 0.4	2.0 ± 0.4	1.8 ± 0.5	0.18
Bulbar redness – Superior	1.6 ± 0.5	1.5 ± 0.3	1.4 ± 0.4	0.31
Bulbar redness – Inferior	1.5 ± 0.4	1.5 ± 0.3	1.4 ± 0.4	0.17
Limbal redness – Nasal	1.8 ± 0.6	1.7 ± 0.5	1.6 ± 0.5	0.58
Limbal redness – Temporal	1.6 ± 0.6	1.5 ± 0.5	1.5 ± 0.5	0.83
Limbal redness – Superior	1.9 ± 0.5	1.8 ± 0.4	1.7 ± 0.5	0.36
Limbal redness – Inferior	1.9 ± 0.6	1.8 ± 0.5	1.8 ± 0.5	0.77
Corneal staining – Extent (worst case)	1.1 ± 1.0	0.6 ± 0.7	0.5 ± 0.5	***<0.01***
Conjunctival staining – Extent (worst case)	1.5 ± 1.1	1.8 ± 1.1	1.7 ± 1.1	0.54
Palpebral redness – Upper lid	1.9 ± 0.5	1.9 ± 0.7	1.8 ± 0.6	0.49
Palpebral roughness – Upper lid	1.1 ± 0.6	0.9 ± 0.6	0.9 ± 0.7	0.41

*, Bold indicates significant difference ($p < 0.05$) between visits; Italics indicates trend ($p < 0.1$) in difference between visits

2.3.2 Subjective responses:

No significant differences were found in subjective comfort or vision between participants' habitual lens care product at baseline, and cleadew™ over the course of the study (**Table 2.4**). However, there was a trend ($p < 0.1$) for participants to report a significant improvement in end of day comfort with cleadew™ at the one month and three-month follow-up visits compared to their habitual lens care product (**Table 2.4**), but also a trend for slightly more itchiness during use of the cleadew™ solution.

Table 2.4: Subjective variables recorded at each visit to the clinic.

	Baseline (n=40)	**1 month (n=40)**	**3 months (n=38)**	**ANOVA**
Subjective rating (0-100)				
Comfort - Insertion	91 ± 9	91 ± 10	92 ± 9	0.88
Vision – Insertion	89 ± 14	94 ± 9	93 ± 9	0.18
Burning/Stinging – Insertion	93 ± 11	93 ± 12	95 ± 7	0.80
Itching – Insertion	96 ± 6	97 ± 5	93 ± 10	*0.09*
Comfort – End of Day	78 ± 25	87 ± 16	85 ± 15	*0.08*
Vision – End of Day	87 ± 17	89 ± 16	90 ± 12	0.76

2.3.3 Contact lens induced adverse events during use of the povidone-iodine solution:

There was no case of solution induced corneal staining with the cleadew™ cleaning and disinfecting system over the course of the study. A total of three adverse events occurred during the study but none were classified as serious adverse events (**Table 2.5**) and all were deemed to be unrelated to the use of the cleadew™ cleaning and disinfecting system based on histories of the events obtained by questioning the participants. There was one possible contact lens-related corneal infiltrative event (**Table 2.5**, Subject 24) but as this participant reported not wearing their contact lenses on the day the symptoms started and did not wear their contact lenses for 2 weeks prior to the final three-month visit, it was deemed unrelated to use of the solution. Nevertheless, should this adverse event be classified as lens related, the rate of corneal infiltrative events per 100 participant months with cleadew™ was 0.83%.

Table 2.5: Adverse events during use of the povidone-iodine solution.

Subject No.	Visit	Diagnosis	Eye	Serious AE	Product related
12	Unscheduled – Post Baseline	Possible allergy or Meibomian gland blockage in upper eyelid	Left	No	No
24	3 month	Probable infiltrative keratitis	Left	No	No
40	Unscheduled – Post 1 month	Bulbar conjunctival laceration	Left	No	No

2.3.4 Lens case contamination

A total of 75 lens cases were collected from the 40 study participants (1st month = 40 and 3rd month = 35). Five subjects did not return their used lens cases at the final three-month visit. Of the 75, 1 subject could not differentiate between the 3-month lens storage case and the 2-month lens storage case, and 1 subject forgot to use a new lens storage case after the 2nd month. Therefore, data for these lens cases were excluded from the analysis. Of 73 lens cases analysed, 22 lens cases (30%) were culture negative (**Figure 2-2**). There was no significant difference in the contamination rates of lens cases or the type of bacteria cultured between the 2 visits (percentage contamination one month = 72.5% and at three-month = 66.7%, $p = 0.617$), hence the data from both visits were pooled for analysis. The eleven cases that were analysed for microbial contamination of different compartments within the case showed that, in each case, if one compartment was contaminated so was another, and the contaminants were at similar levels and of similar colony types (morphology and Gram stain), as has been published previously for other contact lens multipurpose solutions (Willcox *et al*., 2010). Thus, the data were combined, and all data are presented for total lens case contamination.

Of the 51 contaminated lens cases, 21 (42%) lens cases were contaminated with Gram-positive bacteria only, 12 (24%) lens cases with Gram-negative bacteria only and 18 (35%) lens cases contained both Gram-positive and Gram-negative bacteria. Two or more bacterial strains were cultured from 35 (69%) of the 51 contaminated lens storage cases and seven, the maximum cultured, bacterial strains were cultured from one (3%) contaminated lens case. The overall bacterial bioburden of each lens cases was 1.3 ± 1.2 Log (colony forming unit) CFU/case. **Table 2.6** shows the numbers of different types of microorganisms that were isolated from the lens cases and the percentage of

case contamination. The rate of contamination of cases by different microbial types is shown in **Figure 2-3**. *Staphylococcus epidermidis* and *Serratia marcescens* were the most frequently cultured Gram-positive and Gram-negative bacteria respectively from the contaminated lens storage cases (**Table 2.7**). Significantly higher numbers of Gram-negative bacteria were cultured from contaminated lens cases than Gram-positive bacteria ($p < 0.05$). *Staphylococcus aureus* (299,293 ± 422,908 colony forming units [CFU]/case) and *Klebsiella oxytoca* (226,256 ± 319,957 CFU/case) were cultured in the largest numbers for Gram-positive and Gram-negative bacteria respectively. Also, *Staphylococcus* spp. *Micrococcus luteus* and *Acinetobacter radioresistens* were commonly isolated as pair from the same lens cases (**Table 2.8**). Fungi were isolated from 8% of the cases, but no further identification work was undertaken to identify these contaminants.

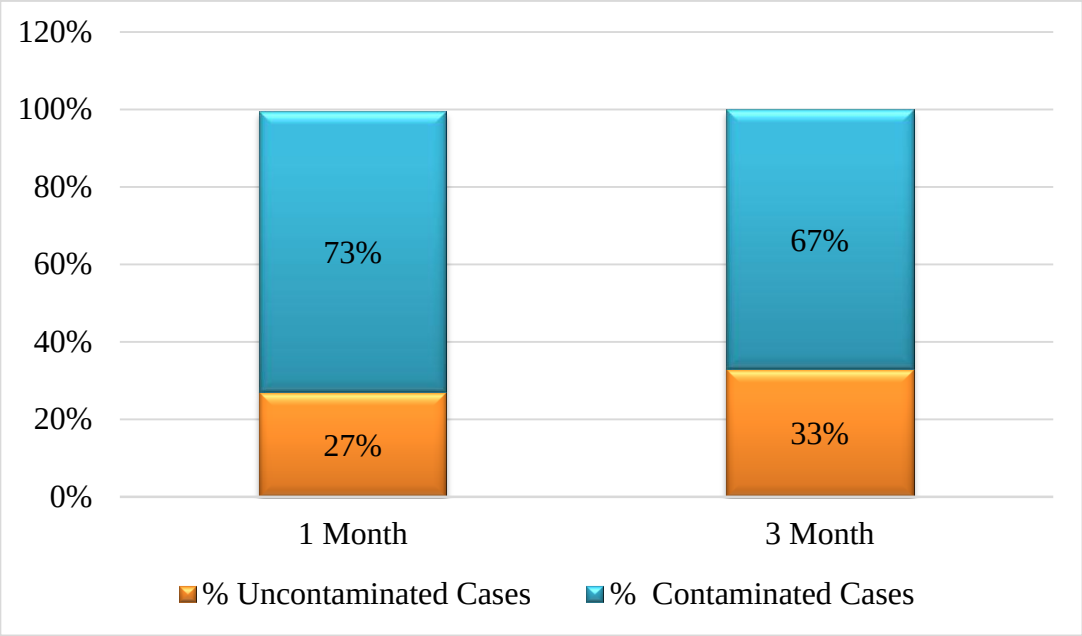

Figure 2-2: The percentage of bacterial contamination of contact lens cases after 1 and 3 months.

Table 2.6: The types of bacteria and their frequency of isolation from contact lens cases.

Visit type	No of organisms	Gram positive	Gram negative
1st month	1 microbial type	17%	42%
	2 microbial types	21%	10%
	> 3 microbial types	10%	_
3rd month	1 microbial type	17%	30%
	2 microbial types	13%	30%
	> 3 microbial types	13%	30%

Table 2.7: Bacterial types isolated from contaminated cleadew™ lens cases.

Bacterial identification		Number of times isolated (% of lens cases)	Colony forming units/case (Mean ± SD)
Gram positive	*Bacillus aquimaris*	1 (1)	1
	Bacillus licheniformis	1 (1)	5
	Brachybacterium rhamnosum	1 (1)	10
	Brevibacterium ammoniilyticum	1 (1)	75
	Enterococcus faecalis	8 (11)	113 ± 149
	Kocuria kristinae	1 (1)	13
	Kocuria palustris	1 (1)	15
	Kocuria spp.	1 (1)	13
	Microbacterium paraoxydans	1 (1)	35
	Microbacterium spp.	1 (1)	1
	Micrococcus luteus	5 (7)	119,062 ± 266,058
	Micrococcus spp.	6 (8)	69 ± 93
	Propionibacterium acnes	4 (5)	10 ± 4
	Staphylococcus aureus	2 (3)	299,293 ± 422,908
	Staphylococcus cohnii	1 (1)	20
	Staphylococcus epidermidis	9 (12)	33 ± 22
	Staphylococcus haemolyticus	3 (4)	38 ± 27
	Staphylococcus pasteuri	5 (7)	65 ± 99
	Staphylococcus saprophyticus	3 (4)	46 ± 38
	Staphylococcus spp.	10 (13)	20 ± 20
	Staphylococcus warneri	8 (11)	38 ± 41
Gram negative	*Acinetobacter radioresistens*	2 (3)	8 ± 4
	Acinetobacter spp.	5 (7)	87,012 ± 194,531
	Citrobacter freundii	1 (1)	4783333
	Citrobacter spp.	1 (1)	428
	Enterobacter spp.	5 (7)	81,214 ± 181,002
	Flavobacterium lindanitolerans	1 (1)	5
	Klebsiella oxytoca	2 (3)	226,256 ± 319,957
	Pantoea anthophila	1 (1)	8
	Pantoea spp.	3 (4)	173 ± 274
	Pseudomonas aeruginosa	2 (3)	120 ± 163
	Pseudomonas japonica	1 (1)	240
	Pseudomonas spp.	3 (4)	32 ± 8
	Raoultella ornithinolytica	1 (1)	350000
	Serratia marcescens	9 (12)	43,047 ± 128,857
	Serratia spp.	2 (3)	94 ± 126

Table 2.8: Gram positive and Gram negative bacteria isolated from lens cases together as a pair.

Bacteria grown together	
Gram positive	**Gram negative**
Staphylococcus spp.	*Serratia marcescens*
Micrococcus spp.	*Acinetobacter* spp.
Enterococcus spp.	*Pseudomonas aeruginosa*

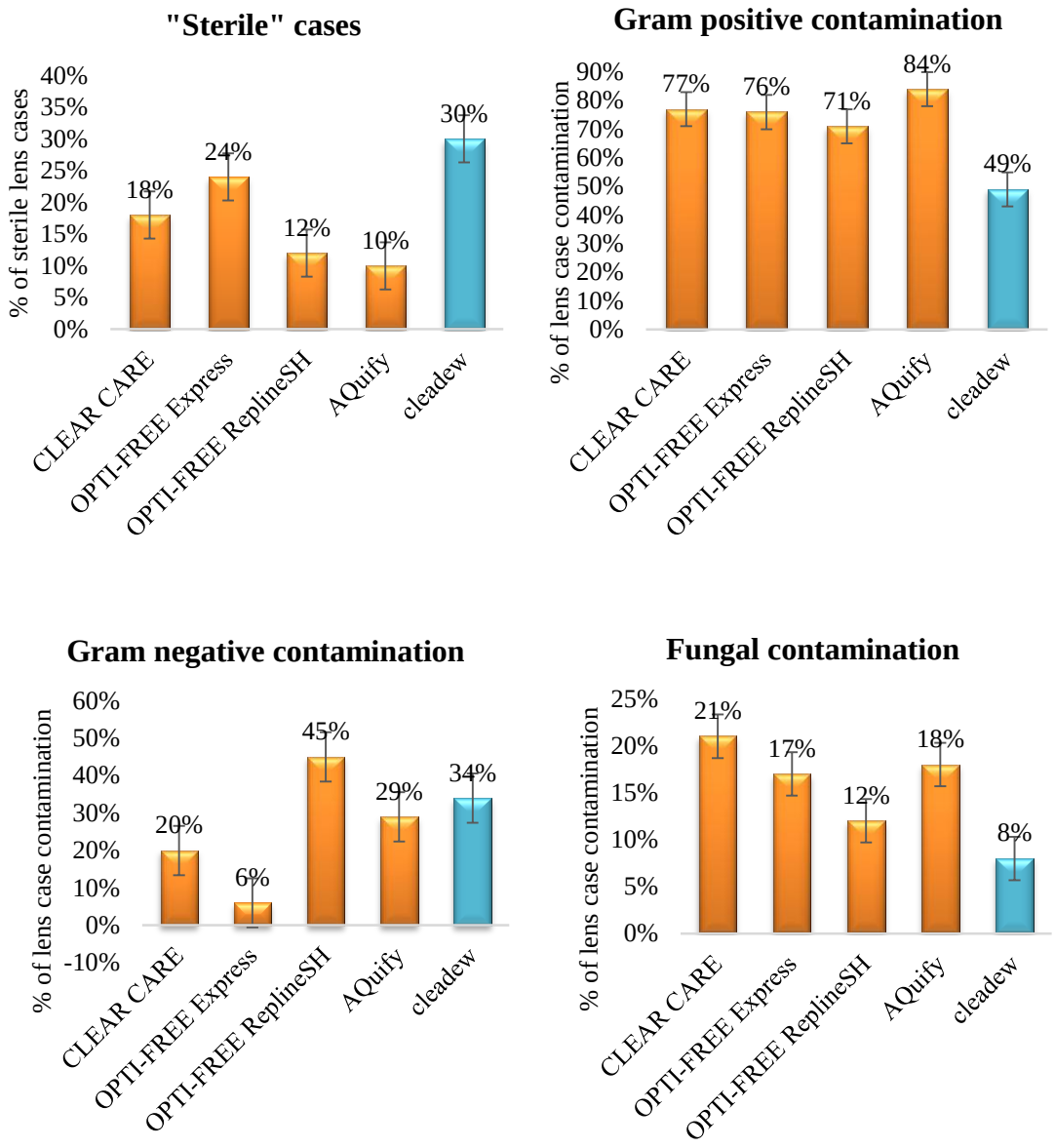

Figure 2-3: Microbial contamination rates of cleadew™ compared to previously published age matched data (Tan *et al*., 2017; Willcox *et al*., 2010).

2.4 DISCUSSION

This study evaluated the cleadew™ cleaning and disinfecting system in daily wear silicone hydrogel and hydrogel CL wearers over 3 months of use. Cleadew™ was well tolerated during contact lens wear, was associated with very low levels of corneal infiltrative events and no cases of solution-induced corneal staining occurred over 3 months of daily wear. This is the first clinical study evaluating the rate of microbial contamination of contact lens cases using a povidone-iodine disinfection system. Approximately 30% of the lens storage cases were sterile and, of the contaminated lens cases, approximately 65% were contaminated by a single microbial type.

This study is the first to report the rates of contamination of worn contact lens cases while using a povidone-iodine based disinfectant. Nearly 70% of the lens cases in this study were contaminated following use, which is similar to studies that have been conducted with currently available single and dual disinfectant or hydrogen peroxide based multi-purpose solutions using similar clinical trial protocols (Tilia *et al*., 2014; Willcox *et al*., 2010), or a recent report of participants in a trial where contact lens cases were cultured after two weeks of use (Dantam *et al*., 2016).

As the clinical trial was run under almost the same protocol as others that have been published, with the exception of not using a defined contact lens type (Carnt *et al*., 2009), and contact lens type did not affect the frequency of contamination of cases during use (Willcox *et al*., 2010), the data from the current study has been compared to previously reported data. This analysis used a test of proportions to compare between levels of contamination of lens cases (**Figure 2-3**). The data demonstrated that the level of uncontaminated (sterile) lens cases with cleadew™ was significantly higher than cases from participants using OPTI-FREE® RepleniSH® (p = 0.0128; Alcon,

Fort Worth, TX, USA), AQuify® ($p < 0.0001$; CIBA Vision, Atlanta, GA, USA) or the hydrogen peroxide solution CLEAR CARE® ($p = 0.0151$; Alcon) but not less than when using OPTI-FREE® Express® ($p = 0.2$; Alcon). The frequency of contamination of lens cases by Gram-positive bacteria with cleadew™ was significantly less than with OPTI-FREE® Express® ($p = 0.004$), AQuify® ($p < 0.0001$) or CLEAR CARE® ($p = 0.001$) but not less than when using OPTI-FREE® RepleniSH® ($p = 0.06$). The level of contamination of cases by fungi when using any of the disinfecting solutions is low, but there was significantly less fungi cultured from cleadew™ compared to CLEAR CARE® lens cases ($p = 0.0385$) only. Conversely, the frequency of contamination of cleadew™ cases by Gram-negative bacteria was significantly higher than OPTI-FREE® Express® ($p < 0.0001$) or CLEAR CARE® ($p = 0.009$) but not OPTI-FREE® RepleniSH® ($p = 0.2$) or AQuify® ($p = 0.3$). When microorganisms isolated from the lens cases was classified as significant or non-significant based on their assumed pathogenicity (Willcox *et al*., 2010), 73% of the contaminated lens cases were found to have non-significant levels of microbial contaminants. These results are significantly better ($p = 0.02$) than the 51% non-significant contamination rate of lens cases with OPTI-FREE® RepleniSH®, but not other lens cases (62 - 85%) (Willcox *et al*., 2010).

Willcox *et al*. demonstrated that the most common Gram-positive bacteria to contaminate contact lens cases were *Staphylococcus epidermidis* (32 - 61% of cases), *Staphylococcus saprophyticus* (18 - 44%), *Staphylococcus aureus* (0 - 9%), and "viridans" streptococci (3 - 13%), whereas the commonest Gram-negative bacteria were *Delftia acidovorans* (1 - 26%), *Stenotrophomonas maltophilia* (2 - 14%), *Serratia marcescens* (0 - 5%) and *Achromobacter* sp. (0 - 10%). Cleadew™ cases were contaminated with *Staphylococcus epidermidis* (12%, $p = 0.007$) or *Staphylococcus*

saprophyticus (4%; $p < 0.0001$) less frequently than other lens cases (Willcox *et al*., 2010). There was no significant difference in contamination with *S. aureus* but cleadew™ cases were contaminated less frequently with viridans streptococci than those of OPTI-FREE® Express® only ($p = 0.005$). Cleadew™ cases were contaminated less frequently with *D. acidovorans* ($p < 0.0001$) or *S. maltophilia* ($p = 0.0011$) than OPTI-FREE® RepleniSH® only. However, cleadew™ cases were contaminated more frequently with *S. marcescens* or *Achromobacter* sp. than OPTI-FREE® Express® ($p = 0.003$ and 0.02, respectively), CLEAR CARE® ($p = 0.005$ and 0.02 respectively), or AQuify® ($p = 0.0023$ *Achromobacter* only). It should be noted that, the technique to identify the different bacterial types in the current study (16S rRNA) was different from the traditional culture techniques used by Willcox *et al*. and this may have affected the results for different species, but not for overall levels of Gram-positive or Gram-negative bacteria as those methods were identical between the two studies (i.e. culture and Gram stain) (Willcox *et al*., 2010).

It is intriguing that there was such a low level of corneal infiltrative events with the use of cleadew™ given the relatively high frequency of contamination of cases by Gram-negative bacteria. A previous study has shown a correlation between the frequency of contamination of lens cases by at least 1 or 3 Gram-negative bacteria (*D. acidovorans, S. maltophilia, S. marcescens*) and the number of corneal infiltrative events (Wiley *et al*., 2012). Perhaps the reason for the low level of corneal infiltrative events with cleadew™ was the very low frequency of contamination with *D. acidovorans, S. maltophilia,* even though there was a relatively high frequency of contamination with *S. marcescens*. Another possible explanation involves the volume of solution in the lens cases. The cleadew™ lens case can hold a volume of up to 8 mL of solution, whereas the volume that can be accommodated in regular flat bottom lens cases of

multipurpose solutions such as those used in the study by Willcox *et al*. (Willcox *et al*., 2010) (e.g. OPTI-FREE® products) is a maximum of 4 mL. This volume difference may dilute the bacteria (or products such as lipopolysaccharide) such that they are not in high enough levels to cause corneal infiltrative events.

Specific combinations of contact lenses and lens care products have been implicated in causing greater levels of corneal staining in both short-term evaluations after two to four hours of lens wear (Andrasko & Ryen, 2008), and in studies of up to three months daily wear (Carnt *et al*., 2009). In this study, no cases of solution induced corneal staining were observed with cleadew™ in silicone hydrogel and hydrogel lens wearers over the three-month period. This shows that the cleadew™ cleaning and disinfecting system has potential health benefits, given that solution induced corneal staining has been potentially associated with a three times increased risk of corneal inflammatory events (Carnt *et al*., 2007). Indeed, only a single possible contact lens-related corneal infiltrative event occurred during the study with cleadew™ (0.8% rate of corneal infiltrative events per 100 participant months). This rate is lower than those reported for Polyquad (3.6%) and PHMB multipurpose solutions (4.2%) and for a one-step hydrogen peroxide based system (2.2%), and is comparable to daily disposable lens wear (0%) (Carnt *et al*., 2007; Jara-de la *et al*., 2013).

Participants tended to report a clinically significant improvement in end of day comfort with cleadew™ at the 1 month and 3 month follow-up visits (average score 85 to 87) compared to their habitual lens care product (average score 78). This may be attributed to the lack of solution induced corneal staining with cleadew™, as previous studies have reported lower end of day comfort scores in participants with solution induced corneal staining compared to those without (Jara *et al*., 2013; Diec *et al*., 2012).

Alternately, ocular comfort in symptomatic contact lens wearers over eight hours of lens wear can be improved by the choice of contact lens and lens care product (Tilia *et al*., 2013). The magnitude of the difference between comfort at baseline compared to after one or three months use of cleadew™ was greater than five points (1-100 points scale). The minimum comfort difference that can be discerned by subjects on this scale has been determined to be five points (Papas *et al*., 2011), indicating that, with an increased sample size (50 in each group) there would likely be a statistical difference in comfort between the habitual multipurpose disinfecting solutions used and the cleadew™ solution. Whilst participants tended to report more itching upon lens insertion when using cleadew™ at the three-month visit compared to their habitual lens care product at baseline, the magnitude of difference was less than 5 on a subjective rating scale of 0 to 100 and so may not be clinically significant. To determine whether this difference in itchiness is statistically significant a clinical trial involving approximately 175 participants in each group would need to be conducted.

This study has some limitations and consequences for further study. The study was conducted as an open-label study design which may introduce bias in subjects' responses to the questionnaire. However, subjects completed a range of questions in regard to comfort and vision for various time-points during the day, and the only noteworthy finding was subjective end of day comfort scores. Had subjective bias been the primary causative factor then we may have expected clinically and statistically significant improvements across all of the subjective responses. It may also be of interest to enrol symptomatic subjects into a trial using cleadew™ to determine whether the use of this solution can improve their symptomatology. Discomfort during lens wear is associated with levels of prolactin-induced protein in the tear film (Masoudi *et al*., 2016). It may be valuable to conduct further research to evaluate

whether there are any corresponding changes to tear film proteins with use of the cleadew™ which may be associated with the improved end of day comfort scores.

In conclusion, this chapter reports that the 70% of the lens cases showed bacterial contamination even after using the cleadew™ povidone-iodine based cleaning and disinfecting system. Although, cleadew™ cleaning and disinfecting system is compatible with a variety of silicone hydrogel and hydrogel contact lenses and has the potential to improve subjective end of day comfort. Use of cleadew™ resulted in very low, if any, rates of corneal infiltrative events associated with lens wear. Also, this study identified the types of organisms most commonly isolated from contact lens cases as a pair. Therefore, cleadew™ cleaning and disinfecting system may be of utility, particularly for use amongst symptomatic contact lens wearers, and to reduce the microbial burden and the potential risks of developing associated contact lens related complications.

Chapter 3:
Bacterial Coaggregation amongst the Most Commonly Isolated Bacteria from Contact Lens Cases

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Chapter 3 Bacterial Coaggregation amongst the Most Commonly Isolated Bacteria from Contact Lens Case

3.1 INTRODUCTION

Microorganisms become more resistant to disinfectants upon attachment and biofilm formation on surfaces (Donlan, 2002; Szczotka-Flynn *et al*., 2014) compared with planktonic organisms. Biofilm formation may facilitate eventual transmission to contact lenses and to the ocular surface (Vermeltfoort *et al*., 2008). Bacterial biofilm formation involves several stages including adhesion to a substrate, micro-colony formation, maturation and eventual dispersal. Within the process of adhesion and bacterial micro-colony formation, microbial coaggregation and cohesion may occur and may contribute to biofilm formation and maturation. This can occur by pioneer microbes providing new surfaces (their own surface) for other bacteria to adhere to (i.e. via coaggregation) or by the pioneer microbes modifying the surface itself to facilitate adhesion of secondary colonisers. There have been no reports of the cohesion abilities and the mechanism of interaction of bacteria isolated from contact lens cases.

The present chapter aimed to examine the coaggregation pattern, mechanism of interaction and cohesion between the most commonly isolated pathogenic bacteria from contact lens induced corneal infiltrative events, *Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus* and *Staphylococcus epidermidis*. Knowledge of these interactions may aid in the rationale design of strategies to reduce adhesion and biofilm formation by bacteria in contact lens cases.

3.2 METHODS

3.2.1 Microbial strains

Bacterial strains and their sources are listed in **Table 3.1**. Five strains each of *Pseudomonas aeruginosa, Serratia marcescens* and *Staphylococcus aureus* and four strains of *Staphylococcus epidermidis* were used in this study. *Actinomyces naeslundii* and *Streptococcus sanguinis* (**Table 3.1**) isolated from dental plaque were used as positive controls for coaggregation (Cisar *et al*., 1979).

Table 3.1: Bacteria used in the study.

Type of bacteria	Micro-organisms	Sources
Gram negative bacteria	*Serratia marcescens* ATCC 13880	Pond water
	Serratia marcescens 27	Microbial keratitis
	Serratia marcescens 5	Contact lens induced acute red eye
	Serratia marcescens 35	Microbial keratitis
	Serratia marcescens 32	Infiltrative keratitis
	Pseudomonas aeruginosa ATCC 9027	Otic infection
	Pseudomonas aeruginosa 6206	Microbial keratitis
	Pseudomonas aeruginosa 6294	Microbial keratitis
	Pseudomonas aeruginosa Paer1	Contact lens induced acute red eye
	Pseudomonas aeruginosa 149	Cystic fibrosis
Gram positive bacteria	*Staphylococcus aureus* ATCC 6538	Human lesion
	Staphylococcus aureus 31	Contact lens induced peripheral ulcer
	Staphylococcus aureus 38	Microbial keratitis
	Staphylococcus aureus 61	Microbial keratitis
	Staphylococcus aureus 62	Microbial keratitis
	Staphylococcus epidermidis ATCC 35984	Contact lens induced acute red eye
	Staphylococcus epidermidis 19	Contact lens induced acute red eye
	Staphylococcus epidermidis 5	Contact lens induced acute red eye
	Staphylococcus epidermidis NCTC 11047	Contact lens induced acute red eye
Positive control	*Actinomyces naeslundii* ATCC 12104	Dental plaque
	Actinomyces naeslundii t14V	Dental plaque
	Actinomyces naeslundii 17952	Dental plaque
	Streptococcus sanguinis CR2B	Dental plaque

3.2.2 Coaggregation assay

Gram negative bacteria were grown in Luria broth (LB; Sigma-Aldrich, Castle Hill, NSW, Australia) and Gram positive bacteria in brain heart infusion (BHI: Becton, Dickinson Macquarie Park, Australia) for 18 - 24 hours at 37ºC. Bacterial cells were collected by centrifugation at 10,000 x *g* for 15 min at 4ºC and washed three times in coaggregation buffer (1 mM Tris (hydroxymethy) amino methane, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl and 3.1 mM NaN_3 at pH 8.0). After the cells were washed the optical density (OD) of the bacterial strains was adjusted to 1.0 (1×10^9 CFU mL^{-1}) at 660nm using a spectrophotometer (FLUOstar Omega; BMG Labtech, Ortenbeg, Germany). An equal volume (0.2 mL) of two cell suspensions was mixed (e.g. *P. aeruginosa* 6294 and *S. aureus* 31), vortexed for 30s and the OD was measured immediately at 660nm (OD1). Then the cell suspension was centrifuged for 2 min at 650 x *g* and then left to stand at ambient temperature for 2 hours and the OD_{660} of 0.2 mL of the upper layer was measured (OD2) (Malik *et al*., 2003). This procedure was repeated again after 24 hours (OD24). The percentage coaggregation was assessed using the following formula:

$$\% \text{ Coaggregation} = \frac{OD1 - OD2 \text{ (or } OD24)}{OD1} \times 100$$

The percentage decrease in optical density was determined and mean and standard deviation were calculated. The experiments were repeated four times. A percentage decrease of greater than 30% indicated coaggregation (Willcox *et al*., 1993).

3.2.3 Inhibition of bacterial coaggregation

Only those coaggregating pairs that showed > 30% coaggregation were tested using inhibitor substances. Lactose and sucrose were used to reverse the coaggregation reactions as described by Cisar *et al*. (Cisar *et al*., 1979). Lactose (0.06 M) or sucrose (0.06 M) were prepared in coaggregation buffer and were individually added to one of the bacterial aggregating pairs (Cookson *et al*., 1995). Individual bacteria from the coaggregation pairs were also treated with protease (pronase from *Streptomyces griseus*; P- 5130, Sigma-Aldrich, Castle Hill, Australia) at a final enzyme concentration of 2mg/mL. The bacterial strains were incubated for 2 hours in lactose, sucrose or pronase at ambient temperature and then the cell suspensions were washed three times with coaggregation buffer to remove unbound inhibitors. The lactose, sucrose or pronase treated bacteria were added with the non-treated bacteria partners and the percentage of coaggregation was recorded after 2 hours of incubation at ambient temperature as described previously. Inhibition of coaggregation was measured by comparing the coaggregation in the absence or presence of the sugars or pronase (Cisar *et al*., 1979; Kolenbrander & Andersen, 1989). A solution of 0.05% Tween-20, 0.2 M NaCl was used as a negative control to control for non-specific bacterial interactions such as those associated with hydrophobicity and ionicity. Only if there was a greater inhibition than this control was inhibition considered to have occurred.

Additionally, the ability of lectin specific sugars (D-galactose and L-fucose) to inhibit coaggregation of *P. aeruginosa* was assessed. *P. aeruginosa* lectins *lecA* (PA-I) and *lecB* (PA-II) bind specifically with D-galactose and L-fucose respectively.(Glick & Garber, 1983) In this assay, *P. aeruginosa* was incubated in coaggregation buffer along

with D-galactose (0.05 M) or L-fucose (0.05 M) at pH 8.0. After washing and incubating with non-treated cells the coaggregation was compared as described above.

3.2.4 Bacterial motility test

A twitching motility assay was performed as described by O'Tolle and Kolter (1998) (O'Tolle & Kolter, 1998). Bacteria were grown as described in the coaggregation assay, washed and resuspended in phosphate buffer saline (PBS; pH 7.4 NaCl 8 g 1^{-1}, KCl 0.2 g 1^{-1}, Na_2HPO_4 1.15 g 1^{-1}, KH_2PO_4 0.2 g 1^{-1} pH 7.2) at an optical density of 1.0 at 660nm. The cells were then stab-inoculated into agar plates (1% [w/v] tryptone; 0.5% [w/v] purified agar, (Oxoid, Basingstoke; UK) 0.5% [w/v] NaCl, (ICN, Biomedicals, Irvine, CA) using a sterile stainless-steel stick and incubated overnight at 37ºC. After incubation the plates were kept at ambient temperature for 2 days. Twitching motility was observed as a faint turbid zone around the stab. The zone and pattern of motility was examined at the bottom of the agar plate. The twitching and swarming motility was differentiated on agar plate by the pattern of their growth. Swarming motility was distinguished from swimming by the appearance of dendritic patterns in the bacterial growth that elongated and branched from a central colony.

Additionally, flagella mediated swimming motility of bacteria was examined using by the hanging drop technique. One drop of sterile PBS was placed on the centre of a glass slide and a loop-full of bacterial suspension grown as previously described was added. A sterile coverslip was placed over the suspension and the coverslip/glass slide edges sealed to maintain humidity. Flagella mediated motility was observed in the drop using a light microscope (Leitz Wetzlar Microscope, Germany) at 200x magnification and care was taken to differentiate active motility from random Brownian motion. The procedure was repeated for three times.

3.2.5 Cohesion assay

The cohesion assay was performed using only the bacterial pairs that showed the highest coaggregation. Strains were grown and adjusted to 0.1 OD_{660} as described in coaggregation assay. *P. aeruginosa* 6294 (2 mL) was added to contact lens storage cases (ReNu® MultiPlus®, Basusch & Lomb, Rochester NY, USA) and the case was loosely recapped and incubated at 37ºC for 24 hours. The lens cases were washed three times with PBS to remove loosely adherent cells and 2 mL of *S. aureus* 31 suspension was then added and incubated at 37ºC for 24 hours. Alternatively, the lens cases were first incubated with *S. aureus* 31 and then with *P. aeruginosa* 6294.

After overnight incubation with the second type of bacteria, lens cases were rinsed gently twice with PBS to remove loosely attached cells. Subsequently, 2 mL of PBS was added to each well of the lens case along with a sterile magnetic stirring bar and the case was vortexed for 1 min to dislodge the adherent bacterial cells. Tenfold serial dilutions of the dislodged bacteria were made in PBS and the dilution were plated on selective isolation agar Cetrimide (Thermo Fisher Scientific, Australia) for *P. aeruginosa* and Mannitol salt agar (Thermo Fisher Scientific, Australia) for *S. aureus*. Plates were incubated for 18 - 24 hours at 37ºC to determine the number of viable bacteria. Controls used a single type of bacterial cell incubated for 24 hours at 37ºC followed by addition 2 mL of sterile PBS and incubation for a further 24 hours at 37ºC and processed as described. The experiments were repeated in three different occasions with three samples each time.

3.2.6 Inhibition of bacterial growth

This assay was performed only on strains used in the cohesion assay. In the first instance, *S. aureus* 31 was grown overnight in BHI and were resuspended to 0.1 OD_{660} in PBS and spread on nutrient agar plate (Thermo Fisher Scientific, Australia) using a sterile cotton swab. Subsequently, 10 µl of *P. aeruginosa* 6294, grown overnight in LB broth and resuspended to 0.1 OD_{660} in PBS, was spotted onto the *S. aureus* lawn and the plate incubated overnight at 37ºC. The same procedure was performed by spotting *S. aureus* culture on a lawn of *P. aeruginosa*. After incubation, inhibition of growth was assessed and graded as follows: ‘‘no inhibition’’ when no zone of inhibition was observed around the bacterial spot of inoculation ‘‘weak inhibition’’ was indicated by an inhibition halo ≤ 6 mm around the bacterial spot inoculation; ‘‘strong inhibition’’ was indicated by an inhibition halo > 6 mm and ≤ 16 mm and ‘‘very strong inhibition’’ was indicated by an inhibition halo > 16 mm (Baldan *et al*., 2014). The inhibitory zone was expressed in mm from three independent experiments measured directly on the agar plates.

3.2.7 Statistical analysis

Data analysis was performed using Microsoft Excel 2010 and Statistical Package for Social Science for Windows version 20.0 (SPSS, Inc, Chicago, IL). The percentage of bacterial coaggregation between different pairs and groups was compared using ANOVA. Additionally, the comparison of the percentage of coaggregation between different time-points was performed using paired sample t-test.

3.3 RESULTS

3.3.1 Coaggregation:

Bacterial coaggregation occurred between different bacterial pairs. In most cases where coaggregation was observed it occurred after two hours of incubation and was only slightly increased at 24 hours. Four out of five *S. aureus* and three out of four *S. epidermidis* strains were able to coaggregate with some of the *P. aeruginosa* strains after 24 hours incubation. *P. aeruginosa* 6294 coaggregated with all strains of *S. aureus*, *P. aeruginosa* 6206 coaggregated with all *S. aureus* strains except ATCC 6538, *P. aeruginosa* coaggregated with *S. aureus* strains 31, 38 and 62, and *P. aeruginosa* only coaggregated with *S. aureus* strain 31 (**Table 3.2**). The highest percentage coaggregation (62% ± 3) was observed between *P. aeruginosa* 6294 and *S. aureus* 31 after 24 hours incubation. *P. aeruginosa* 149 did not coaggregate with any strain of *S. aureus* and *P. aeruginosa* showed a weak coaggregation with only *S. aureus* 31 after 24 hours incubation. *P. aeruginosa* (6294 & 6206) showed significant coaggregation ($p < 0.001$) with *S. epidermidis* strains ATCC 35984 & NCTC 11047 and the maximum percentage of coaggregation was 49% ± 2 between *P. aeruginosa* 6294 and *S. epidermidis* ATCC 35984 after 24 hours. *P. aeruginosa* Paer1 showed significant coaggregation with S. epidermidis NCTC 11047 only. *Serratia marcescens (*ATCC 13880, 27, 5 and 32) showed low and sporadic coaggregation (> 30%; $p < 0.05$) with *S. aureus* (31, 38, 61 and 62) after 24 hours incubation but their percentage of coaggregation did not exceed 33% (**Table 3.2**). Only *S. marcescens* 35 coaggregated with *S. epidermidis* giving a low level (30%) coaggregation with *S. epidermidis* ATCC 35984 after 2 or 24 hours incubation. There was no coaggregation between the two Gram negative bacteria *P. aeruginosa* and *S. marcescens* (data not

shown). However, the two Gram positive bacteria showed coaggregation. *S.* aureus strains 31 & 38 showed significant coaggregation ($p < 0.001$) after 24 hours with *S. epidermidis* strains ATCC 35984 (52% ± 3; 47% ± 2 respectively; **Figure 3-1**) and NCTC 11047 (46% ± 3; 42% ± 2 respectively; **Figure 3-1**). *S. epidermidis* 19 coaggregated with *S. aureus* 31 and 62 and *S. epidermidis* 5 coaggregated with *S. aureus* 38 after 24 hours. The positive control of three *A. naeslundii* strains and *S. sanguinis* CR2B showed 92% ± 3 coaggregation after 24 hours (**Figure 3-1**).

Table 3.2: Coaggregation between Gram positive and Gram negative bacteria after 2 hours or 24 hours of incubation.

*Positive bacterial coaggregation was considered to be as score of ≥ 30%. "-" = coaggregation was < 30%

Percentage of Coaggregation (After 2 hours of incubation)											
Species		***Pseudomonas aeruginosa***					***Serratia marcescens***				
	Strain	**6294**	**6206**	**ATCC 9027**	**149**	**Paer1**	**ATCC 13880**	**27**	**5**	**35**	**32**
Staphylococcus aureus	31	57 ± 2*	47 ± 2*	-	-	51 ± 2*	-	30 ± 2*	-	-	32 ± 3*
	ATCC 6538	-	-	-	-	-	-	-	-	-	-
	38	36 ± 2*	30 ± 4*	-	-	43 ± 2*	-	-	-	-	-
	61	35 ± 4*	-	-	-	-	-	-	-	-	-
	62	-	-	-	-	-	-	-	-	-	31 ± 3*
Staphylococcus epidermidis	ATCC 35984	45 ± 2*	42 ± 4*	-	-	-	-	-	-	30 ± 2*	-
	5	-	-	-	-	-	-	-	-	-	-
	NCTC 11047	32 ± 3*	34 ± 3*	-	-	41 ± 2*	-	-	-	-	-
	19	-	-	-	-	-	-	-	-	-	-
Percentage of Coaggregation (After 24 hours of incubation)											
Species		***Pseudomonas aeruginosa***					***Serratia marcescens***				
	Strain	**6294**	**6206**	**ATCC 9027**	**149**	**Paer1**	**ATCC 13880**	**27**	**5**	**35**	**32**
Staphylococcus aureus	31	62 ± 3*	51 ± 2*	30 ± 3*	-	58 ± 2*	-	33 ± 3*	-	30 ± 4*	30 ± 3*
	ATCC 6538	30 ± 2*	-	-	-	_	-	-	-	-	-
	38	47 ± 3*	32 ± 2*	-	-	45 ± 3*	-	-	-	-	30 ± 4*
	61	38 ± 4*	35 ± 3*	-	-	_	-	-	30±2*	-	-
	62	35 ± 3*	32 ± 4*	-	-	34 ± 3*	32 ± 2*	-	-	-	31 ± 3*
Staphylococcus epidermidis	ATCC 35984	49 ± 2*	45 ± 3*	30 ± 4*	-	-	-	-	-	30 ± 3*	-
	5	-	-	-	-	-	-	-	-	-	-
	NCTC 11047	42 ± 4*	42 ± 3*	-	-	48 ± 3*	-	-	-	-	-
	19	30 ± 3*	-	-	-	-	-	-	-	-	-

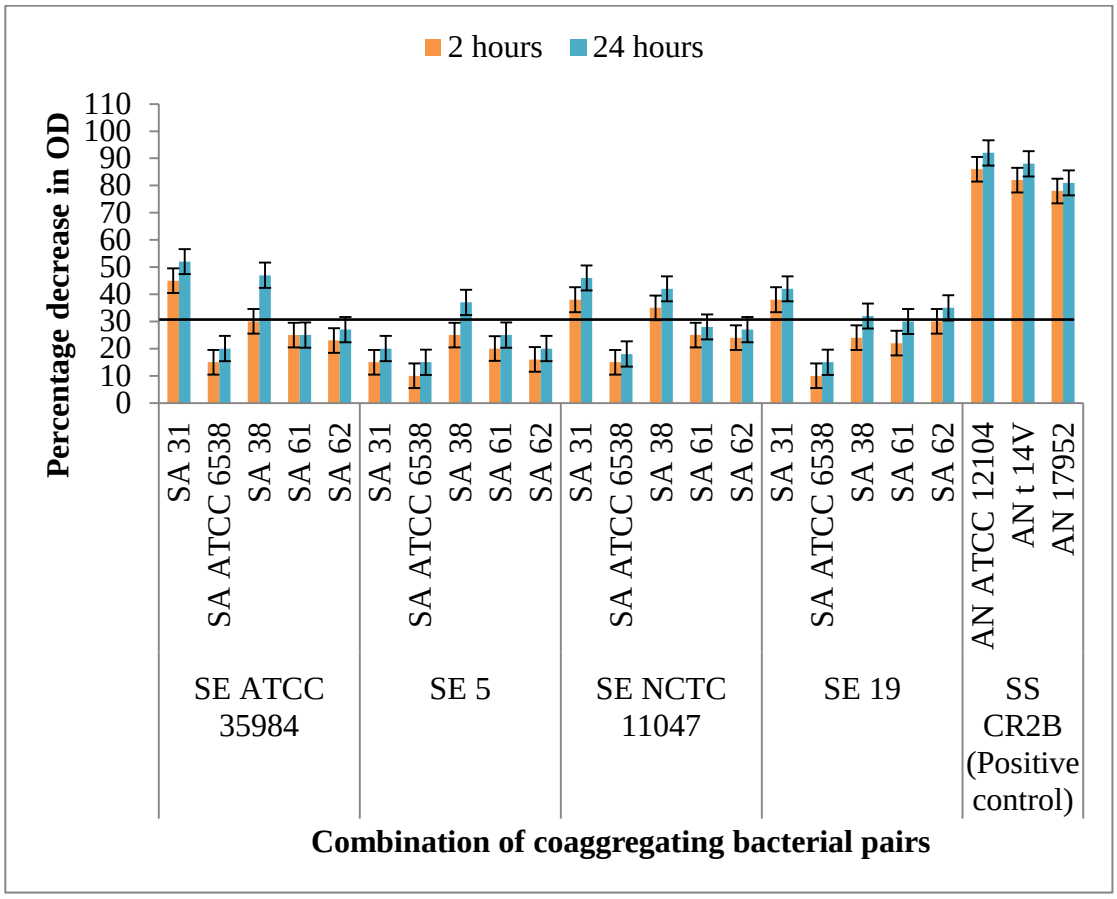


Figure 3-1: Coaggregation between *S. aureus* (SA) and *S. epidermidis* (SE) strains.

Positive coaggregation was considered to be ≥ 30% and is indicated by the line on the graph. Also shown is the percentage coaggregation of the positive control strains: *Actinomyces naeslundii* (AN) with *Streptococcus sanguinis* (SS).

3.3.2 Inhibition of bacterial coaggregation:

The results of the inhibition of coaggregation are shown in **Table 3.3**. The negative control solution of Tween-20 and NaCl (that was used to determine any coaggregation as the result of hydrophobic or charge interactions) gave reductions of coaggregation of between 30% - 38%, therefore a significant inhibition in coaggregation for any treatment was considered to be ≥ 40%. For the coaggregating pairs of *P. aeruginosa* with *S. aureus* or *S. epidermidis*, treatment of *P. aeruginosa* with lactose or sucrose did not inhibit coaggregation (< 40% inhibition), whereas treatment of *S. aureus* or *S. epidermidis* strains with lactose or sucrose inhibited coaggregation ($p < 0.05$) for the most pairs (the exception being *P. aeruginosa* Paer1 and *S. aureus* 38; **Table 3.3**). Lactose or sucrose treatment of *S. aureus* 31 gave 52% ± 3 and 56% ± 4 inhibition of coaggregation with *P. aeruginosa* 6294. When *P. aeruginosa* strains were pre-treated with either D-galactose or L-fucose there were reductions ($p < 0.05$) in coaggregation with all tested strains of *S. aureus* and *S. epidermidis* (**Table 3.4**). Additionally, treatment with pronase inhibited coaggregation between certain pairs. The treatment of *P. aeruginosa* with pronase inhibited coaggregation ($p < 0.05$) with all the staphylococci, but pronase treatment to staphylococci did not inhibit coaggregation (**Table 3.3**).

While coaggregation was observed between *S. aureus* and *S. epidermidis* there was no inhibition of coaggregation when *S. aureus* strains were treated with either lactose or pronase. However, treatment of *S. epidermidis* strains with either lactose or pronase inhibited coaggregation with *S. aureus*. For two out of three pairings, treatment of *S. aureus* with sucrose inhibited coaggregation with *S. epidermidis* but treatment of *S. epidermidis* with sucrose did not inhibit coaggregation with *S. aureus*. The

coaggregation between *S. marcescens* and *S. aureus* was inhibited by pronase treatment but not by treatment with sugars.

Table 3.3: Inhibition of coaggregation inhibited using lactose, sucrose or pronase.

Inhibition was performed only with strains showing coaggregation (**Table 3.2**). *Inhibition of coaggregation was considered to have occurred if there was ≥ 40% inhibition of coaggregation.

Bacterial coaggregation pairs†	Coaggregation inhibition* (% compared to non-treated controls, mean ± SD) after treatment with: Lactose (0.06M)		Sucrose (0.06M)		Pronase (2mg/ml)	
	Treatment of:					
	SA or SE	PA or SM	SA or SE	PA or SM	SA or SE	PA or SM
PA 6294 + SA 31	52 ± 2	-	56 ± 2	-	-	50 ± 3
PA 6206 + SA 31	50 ± 3	-	48 ± 2	-	-	46 ± 2
PA Paer1 + SA 31	42 ± 2	-	52 ± 3	-	-	42 ± 2
PA 6294 + SA 38	46 ± 2	-	48 ± 2	-	-	40 ± 2
PA Paer1 + SA 38	-	-	43 ± 2	-	-	41 ± 5
PA 6294 + SA 61	41 ± 2	-	41 ± 4	-	-	40 ± 2
SM 27 + SA 31	-	-	-	-	-	50 ± 2
SM 35 + SA 31	-	-	-	-	-	50 ± 3
PA 6294 + SE ATCC 35984	46 ± 2	-	51 ± 2	-	-	40 ± 3
PA 6206 + SE ATCC 35984	48 ± 2	-	52 ± 5	-	-	50 ± 2
PA 6206 + SE NCTC 11047	49 ± 2	-	49 ± 2	-	-	48 ± 3
PA 6294 + SE NCTC 11047	50 ± 3	-	50 ± 2	-	-	45 ± 3
PA Paer1 + SE NCTC 11047	41 ± 4	-	51 ± 3	-	-	41 ± 3
	SA	SE	SA	SE	SA	SE
SA 31+ SE ATCC 35984	-	46 ± 2	42 ± 2	-	-	47 ± 3
SA 38+ SE ATCC 35984	-	52 ± 3	44 ± 2	-	-	46 ± 2
SA 61+ SE ATCC 35984	-	46 ± 2	-	-	-	49 ± 2
SA 38 + SE 5	-	48 ± 2	47 ± 3	-	-	46 ± 2
SA 38 + SE NCTC 11047	-	43 ± 2	46 ± 2	-	-	-
SA 31 + SE NCTC 11047	-	41 ± 4	-	-	-	41 ± 2
SA 31 + SE 19	-	42 ± 2	-	-	-	-
SA 62 + SE 19	-	44 ± 2	-	-	-	-

Table 3.4: Inhibition of coaggregation inhibited using D-galactose (0.05M) and L-fucose (0.05M).

Coaggregation inhibition was performed only with *P. aeruginosa* strains. Bacterial coaggregation was considered to have occurred in there was ≥ 40% change compared to no treatment.

Bacterial coaggregation pairs	**% inhibition of coaggregation (mean ± SD)**		
	D-galactose (Only tested for *P. aeruginosa*)	**L-fucose (Only tested for *P. aeruginosa*)**	**Tween 20 + NaCl (negative control)**
P. aeruginosa 6294 + *S. aureus* 31	50 ± 2	58 ± 3	38 ± 5
P. aeruginosa 6206 + *S. aureus* 31	41 ± 2	48 ± 2	32 ± 2
P. aeruginosa 6294 + *S. aureus* 38	42 ± 2	55 ± 2	34 ± 2
P. aeruginosa 6294 + *S. epidermidis* ATCC 35984	42 ± 5	55 ± 2	31 ± 3
P. aeruginosa 6206 + *S. epidermidis* ATCC 35984	41 ± 3	53 ± 4	32 ± 2
P. aeruginosa 6206 + *S. epidermidis* NCTC 11047	43 ± 4	55 ± 3	30 ± 4
P. aeruginosa 6294 + *S. epidermidis* NCTC 11047	48 ± 2	57 ± 4	32 ± 5

3.3.3 Bacterial motility:

Type IV pili or flagella may be involved in the coaggregation responses, we sought to determine whether the strains of *P. aeruginosa* and *S. marcescens* possessed these motility-mediating surface structures by assessing the motility patterns of the strains used. All *P. aeruginosa* strains were motile (swimming) by the hanging drop assay and *P. aeruginosa* 6294, 6206 & ATCC 9027 exhibited twitching motility by the agar stab assay (**Figure 3-2**). All *S. marcescens* strains were motile by the hanging drop assay, and three strains (ATCC 13880, 27 and 35) showed swarming motility (**Figure 3-2**). However, there was no overall relationship between of these motility types and the ability to coaggregate.

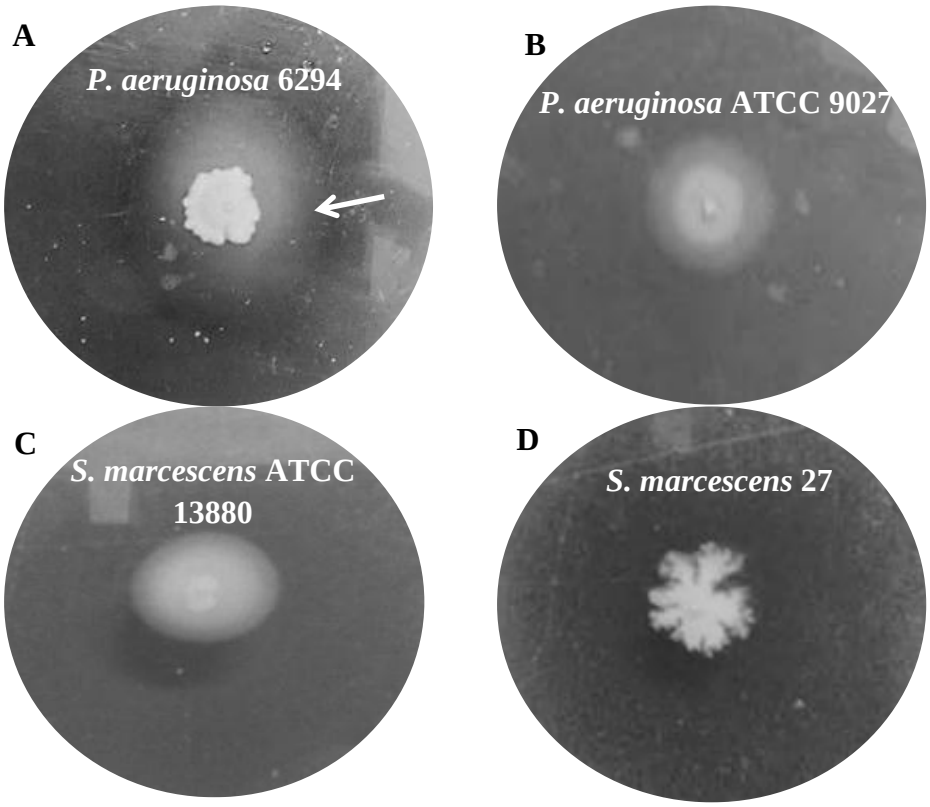


Figure 3-2: Representative images of the twitching and swarming motility of *P. aeruginosa* (6294, ATCC 9027) and *S. marcescens* (ATCC 13880, 27).

(A) & (B) represent the pili-mediated motility of *P. aeruginosa*; the arrow in the picture A indicates a halo zone of pili motility of *P. aeruginosa* 6294 (halo zone: A>B). (C) Represent the swimming motility of *S. marcescens* ATCC 13880 and (D) is the swarming type motility of *S. marcescens* 27 showing a dendritic pattern.

3.3.4 Bacterial cohesion:

S. aureus 31 adhered less (7.6 ± 0.2 $\log_{10}$ CFU; colony forming unit) to the surface of contact lens storage cases compared to *P. aeruginosa* 6294 (8.4 ± 0.1 $\log_{10}$ CFU) when grown in isolation. There was no evidence of positive cohesion between *P. aeruginosa* 6294 and *S. aureus*. When, *S. aureus* 31 and *P. aeruginosa* 6294 were grown together, *S. aureus* 31 was inhibited irrespective of which bacteria was added first. When both *P. aeruginosa* 6294 and *S. aureus* 31 were grown together in lens cases their recovery was reduced 2.8 $\log_{10}$ CFU and 5.0 $\log_{10}$ CFU respectively, compared to their viable count when adhered in isolation (**Figure 3-3**). When these strains were examined for the production of substances that could inhibit each other, there was inhibition of the growth of *S. aureus* when *P. aeruginosa* was spotted (inhibitory zone size 6.0 ± 0.3 mm) but not when *S. aureus* was spotted onto a lawn of *P. aeruginosa*. Representative images of the inhibition for *S. aureus* 31 and *P. aeruginosa* 6294 are shown in **Figure 3-4**.

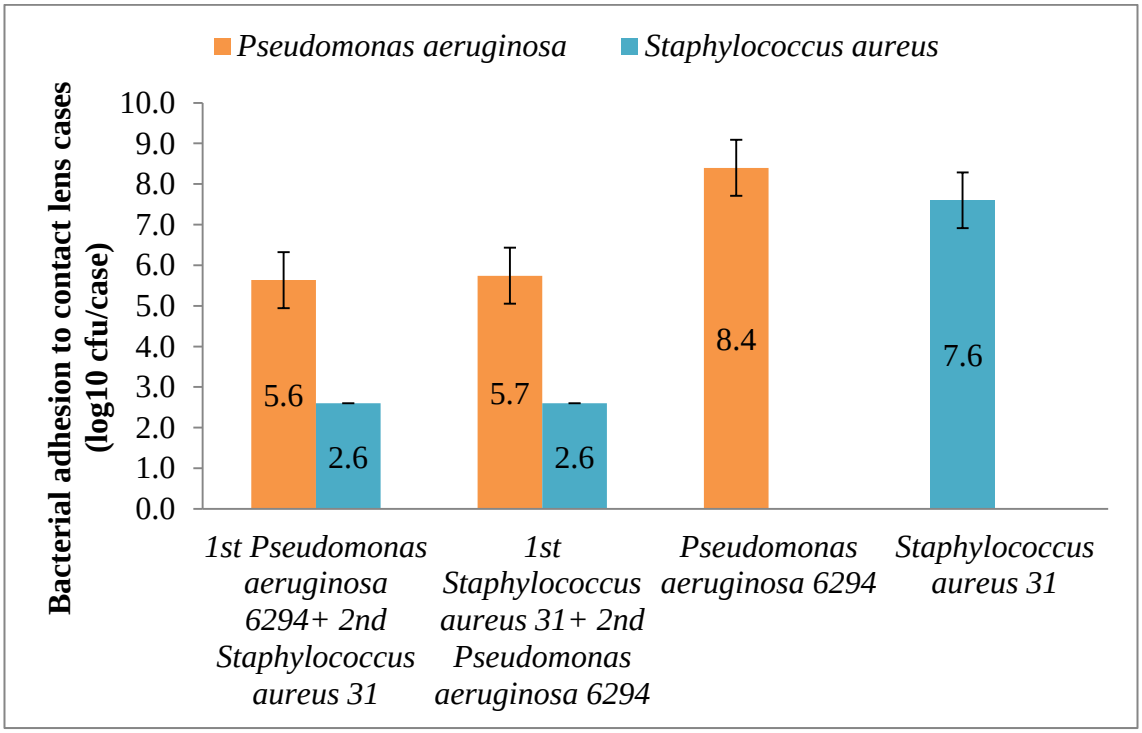


Figure 3-3: Adhesion of *S. aureus* 31 and *P. aeruginosa* 6294 when grown together compared to when grown separately.

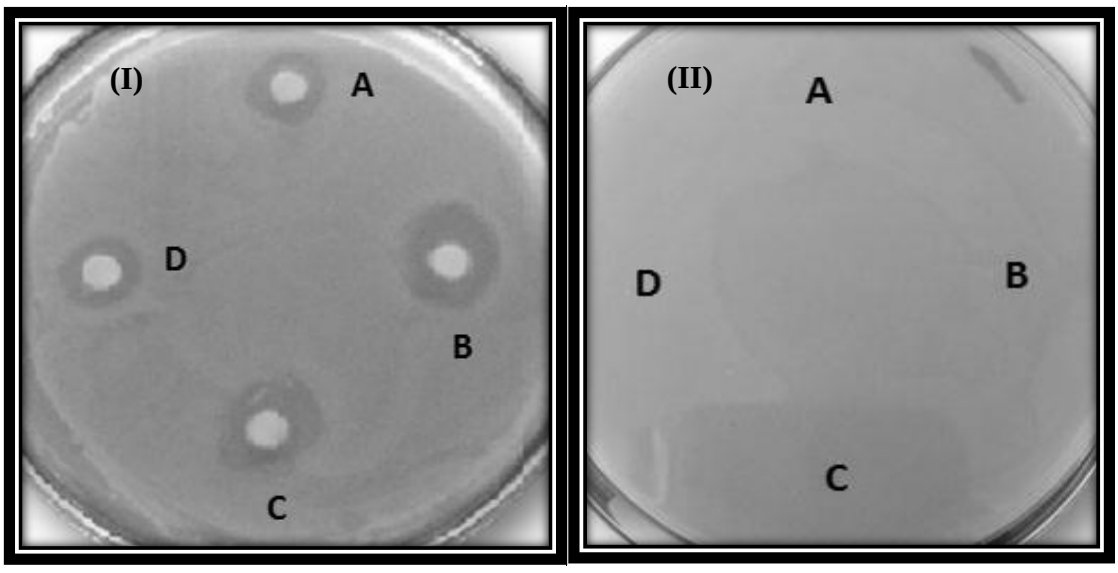


Figure 3-4: Production of inhibitory substances observed as halo zones by *P. aeruginosa* 6294 on a lawn of *S. aureus* 31 (I) or *S. aureus* 31 on a lawn of *P. aeruginosa* 6294 (II).

3.4 DISCUSSION

The primary goal of this investigation was to determine whether bacteria commonly isolated from contact lens cases could coaggregate or cohere. The study demonstrated for the first time that coaggregation occurred between *P. aeruginosa* strains and strains of *S. aureus* isolated from the eye. Two Gram positive bacteria, *S. aureus* and *S. epidermidis*, also showed sporadic coaggregation. *S. marcescens* strains did not exhibit any significant coaggregation with *P. aeruginosa* and exhibited only sporadic low level coaggregation with *S. aureus* only. However, no coaggregating pairs exhibited the high scores given by the positive control paring of *A. naeslundii* and *S. sanguinis*. The coaggregating pairs did not show cohesion, that is, the adhesion of one type of bacteria did not promote the adhesion of the other type from the coaggregating pair. Indeed, this study showed evidence that when grown together *P. aeruginosa* could inhibit the growth of *S. aureus*.

Sato *et al*. demonstrated that coaggregation between actinomycetes and streptococci occurred via lectin-like substances (i.e. substances similar or identical to proteins that bind sugars) on the surface of actinomycetes with carbohydrate(s) on the surface of streptococci (Sato *et al*., 1984). The coaggregation of *Acintomyces oris* with *Streptococcus oralis* is mediated by a single protein on the surface of the actinomycetes named coaggregation factor A (Reardon-Robinson *et al*., 2014) or by proteinaceous type 2 fimbriae on the actinomycetes interacting with carbohydrates on the streptococci (He *et al*., 2011). However, in the current study, pre-treating staphylococci with lactose or sucrose inhibited the coaggregation between them and *P. aeruginosa*. Pre-treating *P. aeruginosa* with pronase also inhibited the coaggregation. This demonstrates that the staphylococci have a substance on their surface, presumably

a lectin that is resistant to digestion by the protease from *Streptomyces griseus*, that interacts with carbohydrates on the surface of *P. aeruginosa* containing structures similar to lactose and sucrose, as well as a protein on the surface of *P. aeruginosa* that mediates coaggregation. When the sugars, fucose or galactose were incubated with *P. aeruginosa* they inhibited coaggregation with the staphylococci. This latter finding implicates the two specific lectin proteins of *P. aeruginosa* LecA and LecB (PA I and PA II respectively) (Winzer *et al*., 2000) presumably binding to the carbohydrates on the surface of the staphylococci. Sucrose is composed of glucose and fructose, lactose is composed of glucose and galactose, galactose and fucose are monosaccharides. LecA has been shown to specifically bind to galactose or glucose, whilst LecB binds specifically to fucose or mannose (Blanchard *et al*., 2014; Sommer *et al*., 2014). In addition, the Type IV pili of *P. aeruginosa* can bind to the b-N-acetylgalactosamine (1–4)-b-galactose via the pilus subunit PilA. *S. aureus* strains can produce various capsular polysaccharides that can coat their surface, and these capsules can contain N-acetylgalactosaminuronic acid, N-acetyl-D-fucosamine, N-acetyl-D-glucosaminuronic acid, and N-acetylmannosaminuronic acid (Riordan & Lee, 2004) which may represent the ligands for the *P. aeruginosa* protein(s). *P. aeruginosa* has a number of surface polysaccharides that may be involved in coaggregation including glycosylated PilA (Nguyen *et al*., 2012), flagella (Schirm *et al*., 2004), and lipopolysacchairde of its outer membrane (King *et al*., 2009) which can have D-glucose, D-galactosamine (King *et al*., 2009), or N-acetyl-D-fucosamine (Castric *et al*., 2001) which are implicated as being potential coaggregation sites based on the inhibition studies reported herein. Thus, in this instance both coaggregating pairs appear to have carbohydrate(s) and protein(s) involved in the coaggregation. Perhaps this is why treating with any of the sugars or pronase only partly reduced coaggregation. The fact that sugars could inhibit

coaggregation may mean that they could be used to reduce multispecies biofilm build-up in contact lens cases. It is unlikely that the simple sugars used in the current investigation would be of use as these can be used as food sources by many different types of bacteria. However, non-metabolizable sugar analogues may be of use to control coaggregation.

Due to the possibilities of type IV pili or flagella being involved in the coaggregation responses, we sought to determine whether the strains of *P. aeruginosa* and *S. marcescens* possessed these motility-mediating surface structures by assessing the motility patterns of the strains used. Type IV pili mediate a form of motility in *P. aeruginosa* known as twitching motility (Burrows, 2012). Flagella mediate both swimming and swarming motility (Harshey & Partridge, 2015). All strains of *P. aeruginosa* and *S. marcescens* showed (flagella-mediated) swimming motility and *S. marcescens* strains ATCC 13880, 27 and 35 showed swarming motility. As only two of the *S. marcescens* strains and only the ocular isolates of *P. aeruginosa* (i.e. 3 strains) showed coaggregation, this demonstrates that possession of flagella *per se* was not associated with coaggregation. Three strains of *P. aeruginosa* (6294, 6206, ATCC 9027) demonstrated twitching motility indicating the possession of functional Type IV pili, but these strains were not necessarily those that showed coaggregation with staphylococci (6294, 6206, Paer1), again indicating that possession of functional Type IV pili is not a requirement for coaggregation. However, as the Type IV pili can be either glycosylated or non-glycosylated and the two types of flagella in *P. aeruginosa* are differently glycosylated further work is required to determine exactly the relationship between motility and coaggregation in these bacteria (Gastric, 1995; Nothaft & Szymanski, 2010).

The concept of coaggregation contributing to the build-up of bacterial communities on surfaces is well established, especially in the dental literature (Jakubovics, 2015). Thus, we sought to determine whether those bacteria that produced coaggregation were associated with cohesion. Cohesion can also occur when a pioneer species modifies the substratum to encourage adhesion of successor species. The most common bacteria found in contact lens cases are staphylococci which may then be the pioneer species. Gram-negative bacteria such as *P. aeruginosa* or *S. marcescens* are much more rarely isolated from lens cases (Szczotka-Flynn *et al*., 2010; Willcox *et al*., 2010) and so can be considered as successor species. The data in the current study demonstrated that although there was evidence of coaggregation between staphylococci and *P. aeruginosa* these bacteria did not cohere. In fact, this study showed evidence for the production of substance(s) by *P. aeruginosa* that inhibited the growth of *S. aureus*. Whilst the nature of the inhibiting substances was not investigated in the current experiments, several inhibitory substances have been reported including DesB (acyl-CoA delta-9-desaturase), 2-heptyl-4-hydroxyquinoline N-oxide and siderophores, and staphylolysin (also called LasA protease) (Barequet *et al*., 2009; Filkins *et al*., 2015; Kim *et al*., 2015b). It may be of value to conduct future experiments on these proteins to determine whether any can prevent the growth of *S. aureus* in contact lens cases. The fact that the production of the inhibitory substance by *P. aeruginosa* did not result in higher adhesion when *P. aeruginosa* was allowed to adhere after *S. aureus* may indicate that death of the *S. aureus* strains does not remove them from the substrata and so does not result in unveiling of any new adhesion sites for *P. aeruginosa*.

In summary, this study demonstrated for the first time that ocular isolates of *P. aeruginosa* and *S. aureus* could coaggregate, but that this may not be related to the

build-up of biofilms in contact lens cases as there was no evidence that the coaggregation was associated with cohesion between the strains. Furthermore, the study confirmed that *P. aeruginosa* can inhibit the growth of *S. aureus.* This chapter evaluated commonly isolated bacterial species from lens cases and subsequent studies will evaluate strains co-existing in contact lens storage cases. The future approach will be to investigate the bacterial interactions and the mechanism in the build-up of biofilm formation by the statins co-existing in contact lens storage cases.

Chapter 4: Interactions between biofilm formation by bacterial species co-isolated contact lens cases

This chapter has been presented as follows:

Paper presentation

- ***Datta A***, Stapleton F, Willcox MDP. (2017) "Understanding bacterial behaviour that may influence biofilm formation in contact lens cases". International Society of Contact Lens Research (ISCLR), *Oregon, United States of America.*

Poster

- ***Datta A***, Stapleton F, Willcox MDP (2018) "Bacterial coaggregation and cohesion among the isolates from contact lens cases". Association for Research in Vision and Ophthalmology (ARVO). *Hawaii, United States of America*.

This chapter has published as:

- ***Datta A,*** Stapleton F, Willcox MDP (2018). Bacterial coaggregation and cohesion among the isolates from contact lens cases. *Invest Ophthalmol Vis Sci*. 2018; 59(7):2729-2735.

Chapter 4 Interactions between biofilm formation by bacterial species co-isolated contact lens cases

4.1 INTRODUCTION

The previous chapter (Chapter 3) explored the bacterial biofilm formation among the ocular isolates commonly isolated from contact lens storage cases demonstrated that ocular isolates of *P. aeruginosa* and *S. aureus* can coaggregate, but their coaggregation was not associated with cohesion between the strains. However, the tested bacterial strains were not co-existing. Chapter 2 was able to detect the co-existing bacterial strains isolated from lens cases. Therefore, this chapter has aimed to understand the role of cohesion, coaggregation and growth in the build-up of biofilm formation of *Staphylococcus* spp. and other commonly isolated co-existing contact lens case contaminants.

4.2 METHOD

4.2.1 Microbial strains

The co-existing bacterial strains of *Staphylococcus epidermidis, Staphylococcus haemolyticus, Micrococcus luteus* and *Acinetobacter radioresistens* (two strains each) isolated from contact lens cases of two asymptomatic wearers, enrolled in a previous clinical trial (Tan *et al*., 2017) were used in this study (**Table 4.1**). *Actinomyces naeslundii* ATCC 12104 and *Streptococcus sanguinis* CR2B, isolated from dental plaque were used as positive controls for coaggregation (Datta, Stapleton, & Willcox, 2017)

Table 4.1: Microorganisms used in this study.

Bacterial strain	Bacterial species	Source†
Gram positive	*Micrococcus luteus* 22-1 *Micrococcus luteus* 14-1 *Staphylococcus epidermidis* 22-1 *Staphylococcus haemolyticus* 14-1	Isolated from contact lens cases from asymptomatic daily wearers (same strain numbers indicate from same lens case)
	Actinomyces naeslundii ATCC 12104 *Streptococcus sanguinis* CR2B	Isolated from dental plaque (Positive control)
Gram negative	*Acinetobacter radioresistens* 22-1 *Acinetobacter radioresistens* 14-1	Isolated from contact lens cases from asymptomatic daily wearers (same strain numbers indicate from same lens case)

†, isolates with the same strain numbers were isolated from the same contact lens case.

4.2.2 Cohesion assay

Bacterial cells were grown in brain heart infusion (BHI; Becton Dickinson, Macquarie Park, Australia) for 18-24 hours at 37ºC followed by washing and adjusting the optical density (OD) in phosphate buffer saline (PBS; pH 7.4 NaCl 8 g 1^{-1}, KCl 0.2 g 1^{-1}, Na_2HPO_4 1.15 g 1^{-1}, KH_2PO_4 0.2 g 1^{-1} pH 7.2) to an OD of 0.1 at 660nm (1 x 10^8 CFU/mL) using a spectrophotometer (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). Contact lens cases (ReNu® MultiPlus®, Basusch & Lomb, Rochester NY, USA) were incubated at 37ºC sequentially with two different types of bacteria, each for 24 hours followed by washing the lens cases once with PBS to remove the loosely attached cells. Subsequently, 2 mL of PBS was added to each well of the lens case along with a sterile magnetic stirring bar and the case was vortexed for 1 min to

dislodge the adherent bacterial cells. The recovery of bacterial species was assessed using a selective bacterial growth medium, as described previously in Chapter 3, (Datta *et al*., 2017) Controls used a single bacterial type incubated for 24 hours followed by the addition of sterile PBS and further incubation of 24 hours.

The total number of adherent bacteria was measured after growth at 37ºC for 24 hours on nutrient agar plates (NA; Thermo Fisher Scientific, Australia). The growth of *Staphylococcus* and *Micrococcus* spp. was estimated by growth for 24 hours at 37ºC on the selective medium mannitol salt agar (MSA; Thermo Fisher Scientific, Australia). The recovery of *A. radioresistens* was calculated by assessing the difference in the number of bacteria recovered from MSA and NA plates (*A. radioresistens* + *Staphylococcus* or *Micrococcus* spp), as *A. radioresistens* does not grow on MSA plates. The differentiation of *M. luteus* and the staphylococci on MSA or NA plates was performed by evaluating the morphological appearance of each recovered bacterial colony. *M. luteus* and staphylococci colonies were differentiated by the pattern of pigmentation, golden-brown and bright yellow respectively. This assay was repeated in duplicate on three different occasions.

The ability of bacterial adhering or cohering to the lens cases to produce biofilms was also evaluated. Extracellular DNA (eDNA) in biofilms was quantified using a previously described protocol (Iyer & Hancock, 2012) using 1 μM SYTOX Green (Invitrogen, Molecular Probes, Australia) at excitation/emission wavelengths of 504/523nm. The amount of eDNA produced by strains grown individually in wells was measured by incubating in SYTOX Green for 2 - 4 minutes, as was the amount of DNA produced when strains were grown in the same well sequentially, as per the

protocol for cohesion. These experiments were repeated in duplicate on three different occasions.

4.2.3 Inhibition of bacterial growth

For this assay only, the bacterial pairs that cohered were tested. In the first instance, bacterial cells were grown overnight in BHI and were resuspended to 0.1 OD_{660} in PBS and spread on NA plate using a sterile cotton swab. Subsequently, the other bacteria of the pair was prepared as above and spotted (10 µl) on the bacterial lawn (Datta *et al*., 2017) The plates were then dried and incubated at 37ºC for 24 hours. Inhibition of growth was seen as an inhibitory zone around the spotted strain. The zone of inhibition was graded on 1-4 scale, as described previously in Chapter 3, (Datta *et al*., 2017)

For inhibition of bacterial growth in nutrient broth, the method of Qin *et al.* was used (Qin *et al*., 2009), with modifications. Overnight cultures in BHI of *A. radioresistens*, *M. luteus* or *Staphylococcus* spp. were centrifuged, washed with PBS and diluted to OD 0.1 at 660nm (1 x 10^8 CFU/mL) in BHI. One millilitre of one bacterial suspension was added to 50 mL of the other partner bacterial suspension and the co-cultures were incubated at 37ºC with shaking at 250 rpm. Every 2 hours, the bacterial suspension was diluted tenfold in PBS and these dilutions were plated onto NA and MSA plates and the number of bacteria was counted after overnight incubation at 37ºC. The bacteria on agar plates were identified by evaluating the morphological appearance of each recovered bacterial colony. The experiment was repeated in duplicates on three different occasions.

4.2.4 Coaggregation assay

Bacterial coaggregation was performed as previously described (Chapter 3) In brief, cells were grown in BHI for 18-24 hours at 37ºC followed by washing and adjusting the OD to 1.0 (1×10^9 CFU/mL) in coaggregation buffer (1 mM Tris (hydroxymethyl) amino methane, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl and 3.1 mM NaN_3 at pH 8.0) (Cisar *et al*., 1979) An equal volume (0.2 mL) of two cell suspensions was mixed (e.g. *A. radioresistens* plus *S. epidermidis*), vortexed and the OD was measured after 24 hours of static incubation at ambient temperature (Malik *et al*., 2003), and the percentage coaggregation was assessed by comparing to single suspensions of each bacteria (Datta *et al*., 2017). A percentage decrease in OD of greater than 30% indicated that coaggregation had occurred (Willcox *et al*., 1993). The experiments were performed in duplicate and repeated three times.

4.2.5 Inhibition of bacterial coaggregation

Lactose (0.06 M) and sucrose (0.06 M) were used to inhibit the coaggregation reactions as described previously (Cisar *et al*., 1979; Cookson *et al*., 1995). Additionally, the inhibition of bacterial coaggregation was assessed after incubating bacterial strains in protease (from *Streptomyces griseus*; P- 5130, Sigma-Aldrich, Castle Hill, Australia) (Chapter 3). The bacterial strains were incubated for 2 hours in lactose, sucrose or pronase at ambient temperature and then the cell suspensions were washed three times with coaggregation buffer to remove unbound inhibitors. Then, the treated bacteria were added to their non-treated bacterial partners and the percentage of coaggregation was recorded after 24 hours of incubation at ambient temperature as described previously (Chapter 3). A solution of 0.05% (w/v) Tween-20, 0.2 M NaCl was used as a negative control, to control for non-specific bacterial interactions such as those associated with hydrophobicity and ionicity. Only if there was a greater

inhibition than this control was inhibition of coaggregation considered to have occurred (Cisar *et al*., 1979; Kolenbrander & Andersen, 1989). The experiments were performed in duplicate and repeated three times.

4.3 STATISTICAL ANALYSIS

Data analysis was performed using Microsoft Excel 2010 and Statistical Package for Social Science for Windows version 20.0 (SPSS, Inc, Chicago, IL). The recovery of bacteria after cohesion or co-culture was compared a two-tailed Student's t test and using repeated measures ANOVA (for different time points). The percentage bacterial coaggregation between different pairs and the inhibition of bacterial coaggregation with the treatment of different inhibitory substances were compared using ANOVA with Bonferroni correction for post-hoc multiple comparisons. Statistical significance level was set at $p < 0.05$.

4.4 RESULTS

There was cohesion between the pairs of *S. epidermidis* or *S. haemolyticus* and *M. luteus* or *A. radioresistens*. Primary adhesion of *M. luteus* or *A. radioresistens* significantly (p = 0.05) enhanced the subsequent adhesion of staphylococci (**Table 4.2**). The greatest effect was seen with the primary adhesion of *A. radioresistens* 22-1 which increased the secondary colonization of *S. epidermidis* 22-1 by 1.8 ± 0.3 Log_{10} colony forming units (CFU) compared to the adhesion of *S. epidermidis* 22-1 in isolation (p = 0.002; **Table 4.2**). Additionally, the primary adhesion of *S. epidermidis* 22-1 increased the secondary colonization of *A. radioresistens* 22-1 by 1.1 ± 0.2 Log_{10} CFU, compared to adhesion of *A. radioresistens* alone (**Table 4.2**). The secondary colonisation by *S. epidermidis* 22-1 significantly (p = 0.008) increased the primary adhesion of *A. radioresistens* 22-1 (p = 0.002) and *M. luteus* 22-1 (p < 0.005). The primary adhesion of *M. luteus* 22-1 or *M. luteus* 14-1 significantly increased the secondary adhesion of *A. radioresistens* 22-1 or 14-1 respectively (**Table 4.2**). The secondary colonisation by *S. haemolyticus* 14-1 significantly (p = 0.005) increased the primary adhesion of *M. luteus* 14-1 and the secondary colonisation *S. epidermidis* 22-1 increased the primary adhesion of *M. luteus* 22-1 (p = 0.05; **Table 4.2**).

In the eDNA assay, there was the evidence of biofilm formation with the largest amount of biofilm for bacteria incubated alone being produced by *S. haemolyticus* 14-1 (**Table 4.3**). When these strains were allowed to cohere the amount of biofilm appear to closely mirror the amount produced by the strains adhered alone, and the amounts produced when strains were in combination did not differ from the amounts produced alone by more than 2 standard deviations.

Table 4.2: Bacterial cohesion on contact lens cases between the strains of Staphylococcus spp. *Micrococcus* spp. and *Acinetobacter* spp. which were isolated from the same contact lens case.

Primary adherer	Secondary coloniser†	Adhesion (Log_{10} CFU/lens case well)	
		Numbers of primary adherer	**Numbers of secondary coloniser**
A. radioresistens 22-1	*S. epidermidis* 22-1	3.20 ± 0.4*	4.53 ± 0.3*
	M. luteus 22-1	2.96 ± 0.3	1.78 ± 0.3
	none	2.53 ± 0.5	-
A. radioresistens 14-1	*S. haemolyticus* 14-1	2.25 ± 0.2	3.46 ± 3*
	M. luteus 14-1	3.68 ± 0.6*	2.20 ± 0.2
	none	2.45 ± 0.4	-
M. luteus 22-1	*S. epidermidis* 22-1	3.49 ± 0.3*	4.05 ± 0.4*
	A. radioresistens 22-1	1.60 ± 0.5	3.11 ± 0.5*
	none	2.60 ± 0.4	-
M. luteus 14-1	*S. haemolyticus* 14-1	3.30 ± 0.5*	4.01 ± 0.3*
	A. radioresistens 14-1	2.25 ± 0.4	3.30 ± 0.4*
	none	2.60 ± 0.5	-
S. epidermidis 22-1	*A. radioresistens* 22-1	2.70 ± 0.4	3.60 ± 0.3*
	M. luteus 22-1	3.70 ± 0.4*	2.60 ± 0.4
	none	2.70 ± 0.4	-
S. haemolyticus 14-1	*A. radioresistens* 14-1	2.74 ± 0.2	3.00 ± 0.3*
	M. luteus 14-1	3.68 ± 0.2*	2.78 ± 0.2
	none	3.11 ± 0.4*	-

†, none represents when PBS was added as secondary suspension. *statistically significant ($p < 0.05$) difference in the bacterial adhesion compared to control.

Table 4.3: Estimation of the amount of biofilm formation (eDNA) produced by strains alone or in combination.

Primary adherer	Secondary coloniser†	Biofilm formation	
		Amount of eDNA	**Estimated amount of eDNA***
A. radioresistens 22-1	*S. epidermidis* 22-1	140 ± 22	(93+91) = 184
	M. luteus 22-1	125 ± 17	(93+64) = 157
	none	93 ± 12	-
A. radioresistens 14-1	*S. haemolyticus* 14-1	218 ± 24	(56+194) = 250
	M. luteus 14-1	117 ± 15	(56+43) = 99
	none	56 ± 14	-
M. luteus 22-1	*S. epidermidis* 22-1	152 ± 22	(64+91) = 155
	A. radioresistens 22-1	124 ± 18	(64+93) = 157
	none	64 ± 9	-
M. luteus 14-1	*S. haemolyticus* 14-1	261 ± 22	(43+194) = 237
	A. radioresistens 14-1	115 ± 12	(43+56) = 99
	none	43 ± 8	-
S. epidermidis 22-1	*A. radioresistens* 22-1	140 ± 18	(91+93) = 184
	M. luteus 22-1	156 ± 15	(91+64) = 155
	none	91 ± 20	-
S. haemolyticus 14-1	*A. radioresistens* 14-1	220 ± 11	(194+56) = 250
	M. luteus 14-1	266 ± 19	(194+43) = 237
	none	194 ± 12	-

*, the estimated amount of biofilm was calculated by adding the amount of eDNA produced by strains when incubated alone.

The ability of bacteria to inhibit one another's growth was first investigated on nutrient agar, but no inhibition with any bacterial pairs was found. The effect of co-culturing two different types of bacteria was genera dependent. Incubating staphylococci with *A. radioresistens* 22-1 or 14-1 increased the numbers of both staphylococci ($p < 0.005$; **Figure 4-1**), and *A. radioresistens* 22-1 or 14-1 ($p < 0.005$; **Figure 4-2**) that grew. Conversely, whilst incubating *S. epidermidis* 22-1 with *M. luteus* 22-1 did not significantly ($p = 0.59$; **Figure 4-1**) increase the numbers of *S. epidermidis*, incubating *S. haemolyticus* 14-1 with *M. luteus* 14-1 reduced the final numbers and the growth rate of *S. haemolyticus* 14-1 ($p < 0.005$; **Figure 4-1**). The co-culture of *M. luteus* 22-1 with *S. epidermidis* 22-1 significantly increased the growth of *M. luteus* ($p = 0.007$; **Figure 4-3**). The co-culture of *M. luteus* 14-1 with *S. haemolyticus* 14-1 significantly altered the growth kinetics of *M. luteus* ($p < 0.005$; **Figure 4-3**), but approximately the same number of cells of *M. luteus* were produced after 24 hours incubation.

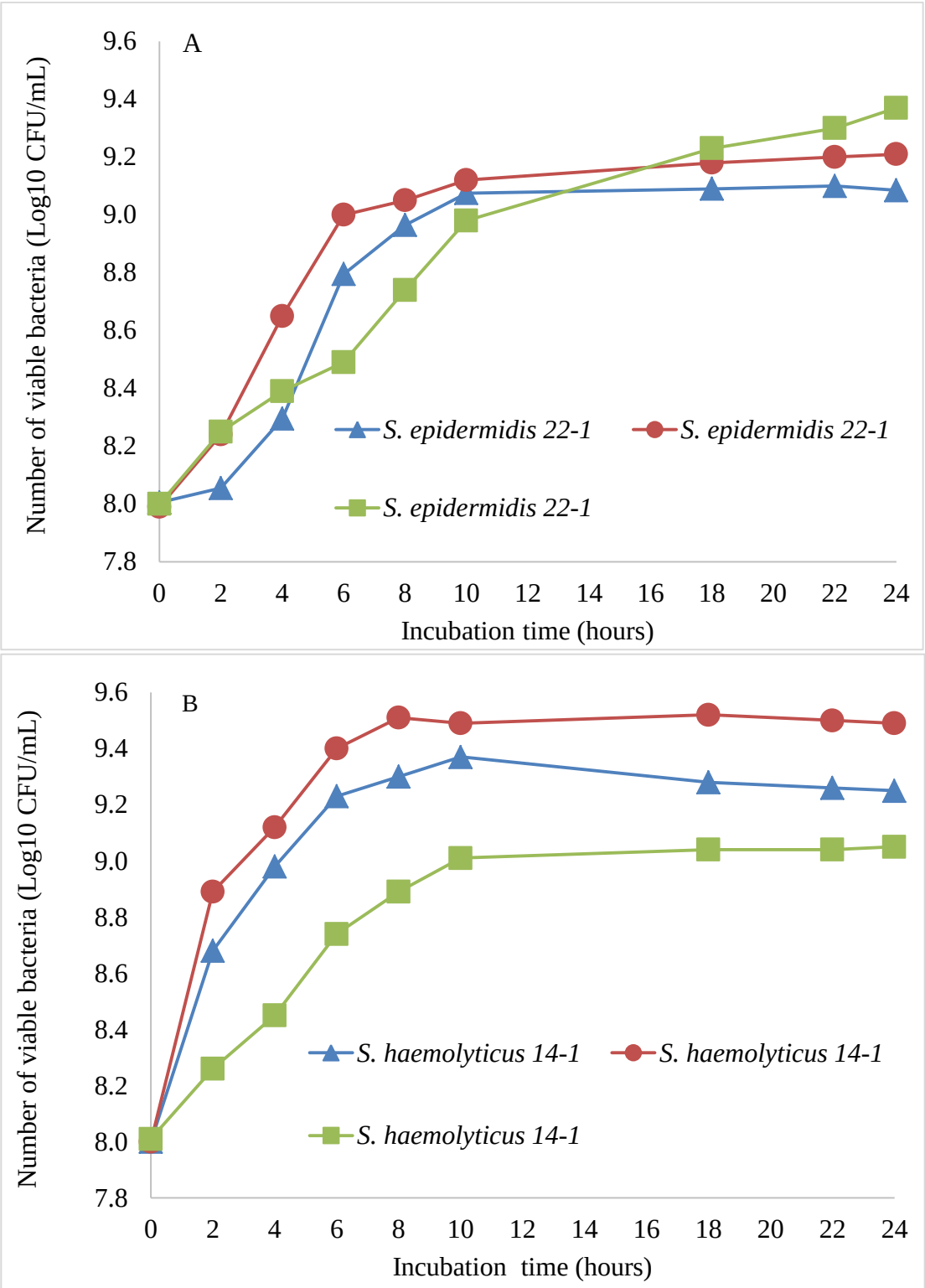


Figure 4-1: Effect of incubation with *A. radioresistans* or *M.luteus* on the growth of *S. epidermidis* (A) or *S. haemolyticus* 14-1 (B). 50 mL of *S. epidermidis* was incubated for 24 hours in BHI alone or in presence of other bacteria.

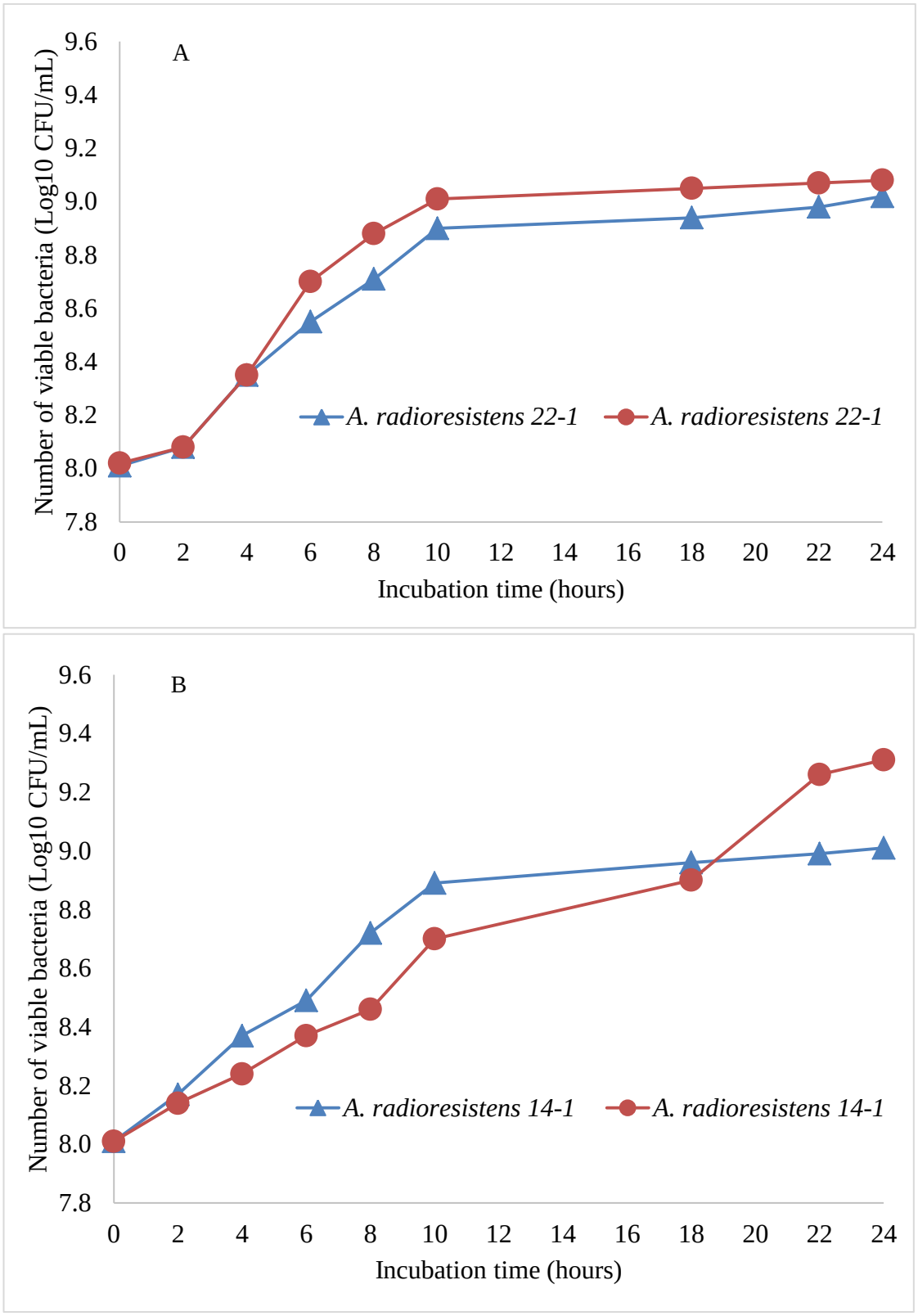
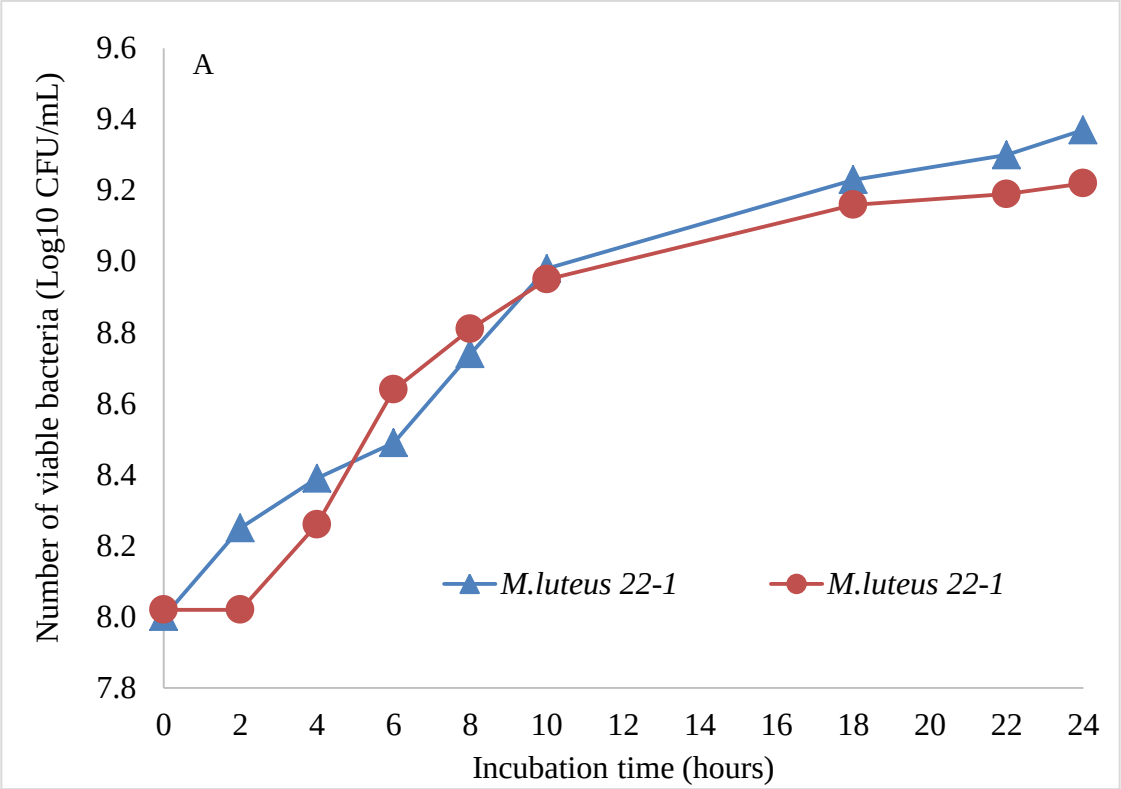


Figure 4-2: Effect of incubation with *S. epidermidis* (A) or *S. haemolyticus* (B) on the growth of *A. radioresistans* strains. 50 mL of *A. radioresistans* was incubated for 24 hours in BHI alone or in presence of *S. epidermidis* or *S. haemolyticus.*



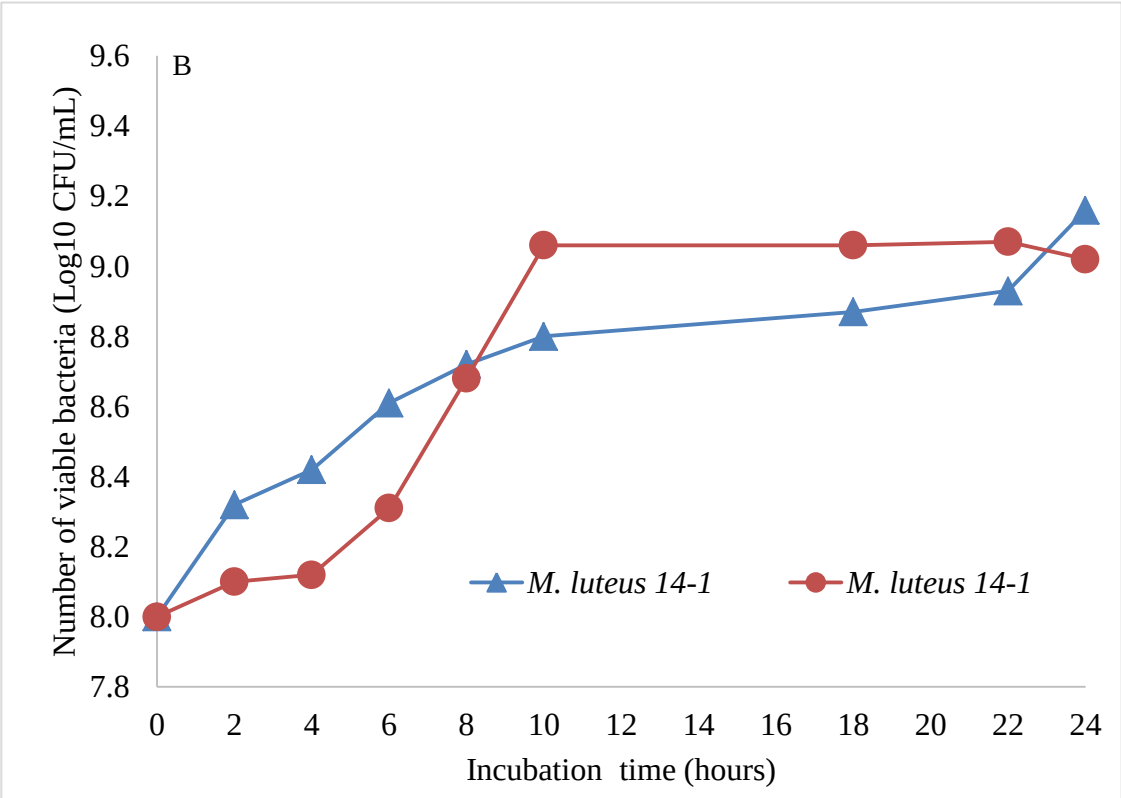


Figure 4-3: Effect of incubation with *S. epidermidis* (A) or *S. haemolyticus* 14-1 (B) on the growth of *M. luteus* strains. 50 mL of *M. luteus* was incubated for 24 hours in BHI alone or in presence of *S. epidermidis.*

Coaggregation was observed between certain strains of *S. epidermidis* or *S. haemolyticus* with *M. luteus* or *A. radioresistens* (**Table 4.4**). The highest coaggregation occurred between *A. radioresistens* 22-1 and *S. epidermidis* 22-1 (54% ± 5; **Table 4.4**). The highest coaggregation between two Gram positive bacteria was 50% ± 3 for the pair *M. luteus* 22-1 and *S. epidermidis* 22-1 (**Table 4.4**). The positive control of *A. naeslundii* ATCC 12104 and *S. sanguinis* CR2B showed 92% ± 3 coaggregation after 24 hours.

The results of the inhibition of coaggregation with lactose, sucrose or pronase are shown in **Table 4.5**. The negative control solution of Tween-20 and NaCl (that was used to determine any coaggregation as the result of hydrophobic or charge interactions) inhibited up to 38% of coaggregation (data not shown), therefore a significant inhibition in coaggregation for any treatment was considered to be ≥ 40% (Willcox *et al*., 1993). Lactose treated *S. epidermidis* 22-1 showed reduced coaggregation with *M. luteus* 22-1 or *A. radioresistens* 22-1 (45% ± 4 and 41% ± 3, respectively). Lactose treatment of *S. haemolyticus* 14-1 caused 42% ± 4 reduction in coaggregation with *A. radioresistens* 22-1. Sucrose treatment of *S. epidermidis* 22-1 reduced coaggregation by 40% ± 3 with *A. radioresistens* 22-1 (**Table 4.5**) only. Incubation of any strain with pronase did not inhibit coaggregation.

Table 4.4: Coaggregation between Gram positive and Gram negative bacteria after overnight incubation. *Positive bacterial coaggregation was considered to be as score of ≥ 30%.

Species	Percentage of coaggregation after overnight incubation	
	***S. epidermidis* 22-1**	***S. haemolyticus* 14-1**
***A. radioresistens* 22-1**	54% ± 5*	48% ± 3*
***A. radioresistens* 14-1**	49% ± 4*	47% ± 2*
***M. luteus* 22-1**	50% ± 3*	44% ± 4*
***M. luteus* 14-1**	41% ± 3*	43% ± 3*

Table 4.5: Inhibition of coaggregation after treatment with lactose (0.06M), sucrose (0.06M) and pronase. *Inhibition of coaggregation was considered to have occurred if there was ≥ 40% inhibition of coaggregation compared to no treatment.

	Percentage of coaggregation that was inhibited by treatment with					
	***S. epidermidis* 22-1**			***S. haemolyticus* 14-1**		
	Lactose	**Sucrose**	**Pronase**	**Lactose**	**Sucrose**	**Pronase**
***A. radioresistens* 22-1**	41% ± 3*	40% ± 3*	20% ± 3	42% ± 4*	32% ± 4	12% ± 4
***A. radioresistens* 14-1**	28% ± 2	22% ± 2	22% ± 2	21% ± 2	11% ± 2	11% ± 2
***M. luteus* 22-1**	45% ± 4*	35% ± 4	25% ± 4	32% ± 4	22% ± 4	12% ± 4
***M. luteus* 14-1**	39% ± 3	25% ± 3	25% ± 3	31% ± 4	21% ± 4	21% ± 4

4.5 DISCUSSION

The current study was designed to understand how multispecies biofilms might form in contact lens cases. Biofilms may be produced by a number of mechanisms and cohesion, coaggregation and stimulation of growth may be important aspects. The present study demonstrated for the first time that *A. radioresistens, M. luteus* and *Staphylococcus* spp. could cohere. Additionally, the presence of *A. radioresistens* increased the growth of *Staphylococcus* spp. Coaggregation could occur between certain strains of *A. radioresistens, M. luteus, S. epidermidis* and *S. haemolyticus* that had been isolated from lens cases of asymptomatic wearers.

No coaggregating pairs presented the high coaggregation scores of the positive controls of *A. naeslundii* ATCC 12104 plus *S. sanguinis* CR2B (92% ± 3). This outcome resembles a previous study finding (Chapter 3) where the coaggregation between *P. aeruginosa* and *S. aureus* reached only (62% ± 3) (Datta *et al*., 2017). In general there was a trend for bacteria isolated from the same contact lens cases to coaggregate with each other, which was similar to the coaggregating pair of *P. aeruginosa* and *S. aureus* in the previous study (Datta *et al*., 2017).

The current study also investigated the inhibitory effect of lactose, sucrose and pronase on coaggregation. Sato *et al.* demonstrated that coaggregation between actinomycetes and streptococci occurred via lectin-like substances (i.e. substances similar or identical to proteins that bind sugars) on the surface of actinomycetes with carbohydrate(s) on the surface of *streptococci* (Sato *et al*., 1984). Pre-treating *Staphylococcus* spp. with lactose or sucrose inhibited the coaggregation which indicates the involvement of staphylococcal lectins in coaggregation, consistent with previous studies (Datta *et al*., 2017; Rickard *et al*., 2004; Rickard *et al*., 2000). However, the selected inhibitory

sugars were unable to stop coaggregation between *Micrococcus* spp. and *Acinetobacter* spp. Understanding how bacteria coaggregate and cohere may help to produce strategies to halt biofilm formation. For example, adding inhibitory substances such as sugars to multipurpose disinfecting solutions may be of benefit. Although, it is unlikely that sugars such as lactose and sucrose can be used as they can be the source of nutrition of many types of bacteria.

Cohesion between the strains of *M. luteus* or *A. radioresistens* with *S. epidermidis or S. haemolyticus* provides support to the concept that the initial adhesion of bacteria may control the secondary colonisation of other types of bacteria (cohesion). *M. luteus* or *A. radioresistens* may form a conditioning film to enhance the adhesion of staphylococci or *vice versa*. However, the amount of biofilm formed by strains, as measured by estimating the amount of eDNA produced, was not affected by them adhering alone or in pairs. The ability of small numbers of *A. radioresistens* 22-1 or staphylococci to stimulate the growth of the other partner in co-culture demonstrates that this can be another factor involved in the cohesion of bacterial cells and hence multispecies biofilm formation. Identifying the factors that are involved in this phenomenon may lead to determining mechanisms to reduce their production or interfere with their mechanism of action, which again could be used to reduce biofilm formation.

In summary, it appears that a complex series of events may take place between bacteria that are involved in multispecies biofilm formation in contact lens cases. Certain bacteria can cohere, that is the presence of one bacterial types increases the ability of another to attach. This can be facilitated by coaggregation, the direct adhesion between cells of different bacteria. Coaggregation may be involved in the cohesion between

stains of *A. radioresistens* or *M. luteus* and staphylococci. The ability of small numbers of *A. radioresistens* or staphylococci to promote each other's growth may also be involved in cohesion and hence biofilm formation. This research will provide a framework for future studies that examine how to reduce biofilm formation in contact lens cases, for example in adding substances to contact lens disinfecting solutions that can prevent aspects of cohesion or coaggregation. This may then reduce the contamination of contact lens cases during use. The research may also have application in other areas. For example, both *Acinetobacter* and *Staphylococcus* can be found concurrently in bronco-alveolar lavages from people with chronic obstructive pulmonary disease (Zakharkina *et al*., 2013). Additionally, the future approach will be to investigate the antimicrobial ability of the antimicrobial lens cases along with contact lens cleaning and disinfecting solution against the bacterial species demonstrated bacterial coaggregation, cohesion or influence in bacterial growth.

Chapter 5:
In vitro Antimicrobial Efficacy of Silver Lens Cases

This chapter has been presented as:

- ***Datta A***. Willcox D, Stapleton F. (2017) "Antimicrobial efficacy of silver copolymerized barrel lens cases". Association for Research in Vision and Ophthalmology (ARVO). *Baltimore, United States of America*.

- ***Datta A***. Willcox D, Stapleton F. (2017) "Antimicrobial efficacy of silver copolymerized barrel lens cases along with disinfecting solution". International Cornea and Contact Lens Congress (ICCLC). *Sydney, Australia*.

Acknowledgemt:

Sauflon Pharmaceuticals provided the silver impregnated contact lens storage cases and Synergi solution during the PhD work.

Chapter 5 In vitro Antimicrobial Efficacy of Silver Lens Cases

5.1 INTRODUCTION

Microbial contamination in lens cases can be associated with the development of contact lens induced ocular infection and inflammation. Commercially available MPDS are the most commonly used disinfecting systems, however they may not be effective at reducing microbial biofilm formation in lens cases (Szczotka-Flynn *et al*., 2014).

Chapter 3 and 4 have demonstrated that certain bacteria can colonise lens cases by coaggregation, cohesion and effect of bacterial growth. This result suggests that there are bacteria which may promote the adhesion and growth of other bacteria in lens cases. The present investigation determines whether new antimicrobial strategies can reduce the adhesion of these bacterial species in lens cases.

Various strategies have been adopted to limit the numbers of bacteria and their biofilm formation on lens cases. Such strategies include the incorporation of silver ions, selenium, polyquaternary ammonium compounds, polymeric pyredium compounds, nitric oxide, furanones and cationic peptides into cases (Reid *et al*., 2013; Weisbarth *et al*., 2007).

Silver ions, often in the form of nanoparticles, provide broad-spectrum antimicrobial activity against Gram positive and Gram negative bacteria, fungi, protozoa and certain viruses (Balazs *et al*., 2004), including antibiotic-resistant strains of bacteria (Gurunathan *et al*., 2014; Stobie *et al*., 2008; Taheri *et al*., 2014). Slow release of silver ions inhibits bacterial growth by multiple methods including enhancing

structural deformities in nucleic acids (Dallas *et al*., 2011), membranes (Abu-youssef *et al*., 2010) and the cell walls of bacteria (Abu-youssef *et al*., 2010; Cavicchioli *et al*., 2010; Dias *et al*., 2006; Ramstedt *et al*., 2007; Sambhy *et al*., 2006). The multiple mechanisms of actions of silver ions may not allow bacteria to easily develop resistance.

Silver-impregnated contact lens cases are commercially available and have been shown to reduce contact lens case contamination (Amos & George, 2006; Dantam *et al*., 2011). Silver-impregnated flat lens cases have robust activity against both Gram positive and Gram negative bacteria (Dantam *et al*., 2012; Dantam *et al*., 2011; Vermeltfoort *et al*., 2008). A silver-impregnated barrel lens case is also available, but their efficacy has not been reported. This study evaluated the *in vitro* antimicrobial activity of silver-impregnated barrel cases compared to non-silver cases in combination with a multipurpose disinfecting solution among the commonly isolated bacteria from lens cases.

5.2 MATERIAL AND METHODS

Strains of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Acinetobacter radioresistens*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus* were used in this study to assess the antimicrobial efficacy of silver barrel lens cases used with contact lens cleaning and disinfecting solution. The details of the strains and their sources are listed in **Table 5.1.**

Table 5.1: Bacterial strains used in the study.

Type of bacteria	Micro-organisms	Sources
Gram negative bacteria	*Serratia marcescens* ATCC 13880	Pond water
	Serratia marcescens 27	Microbial keratitis
	Pseudomonas aeruginosa 6294	Microbial keratitis
	Pseudomonas aeruginosa ATCC 9027	Otic infection
	Acinetobacter radioresistens 22-1	Contact lens case of an asymptomatic wearer
	Acinetobacter radioresistens 14-1	Contact lens case of an asymptomatic wearer
Gram positive bacteria	*Staphylococcus aureus* 31	Contact lens induced peripheral ulcer
	Staphylococcus aureus ATCC 6538	Human lesion
	Staphylococcus epidermidis ATCC 35984	Catheter sepsis
	Staphylococcus epidermidis 22-1	Contact lens case of an asymptomatic wearer
	Micrococcus luteus 22-1	Contact lens case of an asymptomatic wearer
	Micrococcus luteus 14-1	Contact lens case of an asymptomatic wearer

5.2.1 Bacterial culture

Bacteria were revived from the School of Optometry and Vision Science, University of New South Wales culture collection by inoculation into a tryptic soy broth (TSB; Oxoid, Australia) and incubation for 24 hours at 37ºC. Bacterial cells were washed three times by centrifugation at 3000 rpm for 15 min at 25ºC in sterile phosphate buffered saline (PBS; NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L; pH 7.2). The optical density (OD) of the bacterial cell suspensions was adjusted to 0.1 at 660nm (1 x 10^8 colony forming unit/mL [CFU/mL]) in PBS using a spectrophotometer (FLUOstar Omega; BMG Labtech, Ortenberg, Germany) and the cell suspensions were serially diluted to 1 x 10^6 CFU/mL in PBS for all bacteria and in 1:100 TSB for Gram positive or 1:100 LB for Gram negative bacteria (Luria broth; Sigma-Aldrich, Castle Hill, NSW, Australia).

5.2.2 Contact lens case and solutions

Silver barrel lens cases (Sauflon Pharmaceuticals Ltd., London, UK) and non-silver barrel lens cases (control; Sauflon Pharmaceuticals Ltd.) were used. Synergi® disinfecting solution (Sauflon, Pharmaceuticals Ltd.) was used. Synergi® contains an oxychlorite complex (Oxipol™; sodium chloride and hydrogen peroxide), as the disinfecting component. In addition, Synergi® contains polyvinylpyrrolidone and hydroxypropyl methylcellulose as a viscosity enhancing agents (**Table 5.2**).

Table 5.2: Composition of the Synergi® disinfecting solution.

Components in Synergi® disinfecting solution	
Disinfecting agent	Oxychlorite complex (sodium chlorite and hydrogen peroxide)
Buffer	Phosphate
Cheating agent	Not known
Surfactant	Poloxamer
Wetting agent	Polyvinylpyrrolidone (PVP), hydroxypropyl methylcellulose (HPMC)

5.2.3 Antimicrobial activity

Lens cases were opened aseptically and inoculated with 10 mL freshly prepared bacterial suspensions (1 x 10^6 CFU/mL). The lid of the lens cases was loosely recapped, and cases were incubated at 37ºC with agitation (250 rpm) for 24 hours to allow bacterial adhesion.

To test for the antibacterial effects of Synergi®, cases were incubated with the bacterial cell suspension (1 x 10^6 CFU/mL) for 6 hours, 18 hours or 48 hours. Residual bacterial cells were discarded, and cases were rinsed once with PBS to dislodge any planktonic or weakly adherent cells. Following this, lens cases were filled with Synergi® solution (approximately 80% full), recapped and stored at ambient temperature for four to six hours based on the manufacturer's recommended disinfection time. As a control, lens cases were incubated with 10 mL PBS after bacterial adhesion and left at ambient temperature for four to six hours.

5.2.4 Estimation of numbers of bacteria in lens cases

After incubation to allow bacterial adhesion, residual bacteria were discarded, and the cases were rinsed twice with PBS to dislodge any planktonic or weakly adherent cells. Following this, 10 mL of PBS was added to each lens case along with a sterile magnetic stirring bar and the case was vortexed for one minute to dislodge the bacterial cells. Tenfold serial dilutions were conducted in Dey Engley Neutralizing Broth (DE Broth) and dilutions were plated onto trypticase soy agar (TSA, Thermo Fisher Scientific, Australia) containing 0.05% (w/v) Tween 80 (Sigma-Aldrich, Castle Hill, NSW, Australia) and 0.07% (w/v) lecithin (Sigma-Aldrich, Castle Hill, NSW, Australia) and incubated at 37ºC for 18-24 hours for the recovery of bacteria. The numbers of colony forming units were counted and converted to CFU/mL.

5.2.5 Impact of contact lens material and organic soil on disinfection

For testing in the presence of added organic soil, a modified ISO 14729 Stand Alone Test (ISO, 2014) procedure was followed. After bacterial growth in TSB, bacteria were prepared in 0.4% (v/v) organic soil, consisting of fetal bovine serum (0.4% v/v) and of heat-killed *Saccharomyces cerevisia*e OD of 1.5 at 660nm, followed by adjusting the final concentration of bacteria to 1 x 10^6 CFU/mL. Bacteria were incubated in lens cases for 24 hours at 37ºC followed by washing with PBS as described above. Contact lenses were removed aseptically from their packaging, washed three times with PBS and placed in the basket of lens cases. The lens cases were filled with Synergi® disinfecting solution (80% full) and incubated at ambient temperature for 6 hours (the manufacturer's recommended minimum disinfection time), 10 hours or 24 hours. Control lens cases were filled with Synergi® after bacterial adhesion and no contact lenses were added during the disinfection.

After the designated disinfection time, the number of adherent bacteria were evaluated as described above. The number of bacteria adhered to contact lenses was assessed by washing the removed lenses once in PBS, placing into 2 mL of PBS and dislodging the adherent by vortexing in the presence of a small magnetic stirring bar. The resulting lens slurry was diluted in PBS and dilutions plated onto tryptic soy agar containing Tween 80 and lecithin and the colony forming units were enumerated after incubation for 24 hours at 37ºC.

5.2.6 Statistical analysis:

Data analysis was performed using Microsoft Excel 2010 and Statistical Package for Social Science for Windows version 20.0 (SPSS, Inc, Chicago, IL). The total number of viable organisms for each lens case was recorded as Log_{10} CFU/mL. Two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to compare the rate of bacterial recovery from silver and non-silver lens cases. Log differences were summarized as mean ± SD and were compared using a univariate ANOVA for the test organisms at each incubation time. Bonferroni correction was used for post-hoc multiple comparisons.

5.3 RESULTS

5.3.1 Efficacy of silver cases

For Gram positive bacteria, the numbers adherent to silver cases was reduced compared to non-silver cases by an average of 2.61 ± 0.52 Log_{10} CFU/mL when suspended in PBS and 2.82 ± 0.28 Log_{10} CFU/mL when suspended in 1/100 TSB, and these reductions were significant ($p < 0.005$). There was no significant effect of suspending fluid on the activity of silver cases, but more Gram positive bacteria (an average of 1.2 log_{10} CFU/mL) adhered when diluted in 1/100 TSB compared to PBS ($p < 0.005$; **Figure 5-1A**). The maximum inhibition of 3.41 ± 0.06 Log_{10} CFU/mL was observed for *S. aureus* ATCC 6538 ($p = 0.008$) when allowed to adhere in PBS, compared to non-silver barrel cases as a control (**Figure 5-1A**). Within species, there was a significant ($p < 0.005$) difference in the bacterial inhibition between the strains of *S. aureus* (ATCC 6538 & 31) and *S. epidermidis* (ATCC 35984 & 22-1) in lens cases, but not for strains of *M. luteus* (**Figure 5-1A**).

The effect of silver was greater for Gram positive bacterial strains ($p = 0.04$) compared to Gram negative bacteria. For Gram negative bacteria, the numbers adherent to lens cases were reduced on average by 2.36 ± 0.11 Log_{10} CFU/mL when diluted in PBS and 2.71 ± 0.3 Log_{10} CFU/mL when diluted in 1/100 LB, and these reductions were significant ($p < 0.005$). There was no significant effect of diluent on the activity of silver cases, but more Gram negative bacteria (an average of 0.8 Log_{10} CFU/mL) adhered when diluted in 1/100 LB compared to PBS ($p = 0.009$; **Figure 5-1B**). For Gram negative bacteria, a maximum of 3.21 ± 0.02 Log_{10} CFU/mL reduction in adhesion was seen with *A. radioresistens* 22-1 when prepared in 1/100 LB (**Figure 5-1B**). Within species, there were a significant ($p = 0.006$) differences in adhesion

between the strains of *P. aeruginosa* (ATCC 9027 & 6294) and *S. marcescens* (ATCC 13880 & 27), but not for strains of *A. radioresistens* (**Figure 5-1B**), irrespective of the presence of silver.

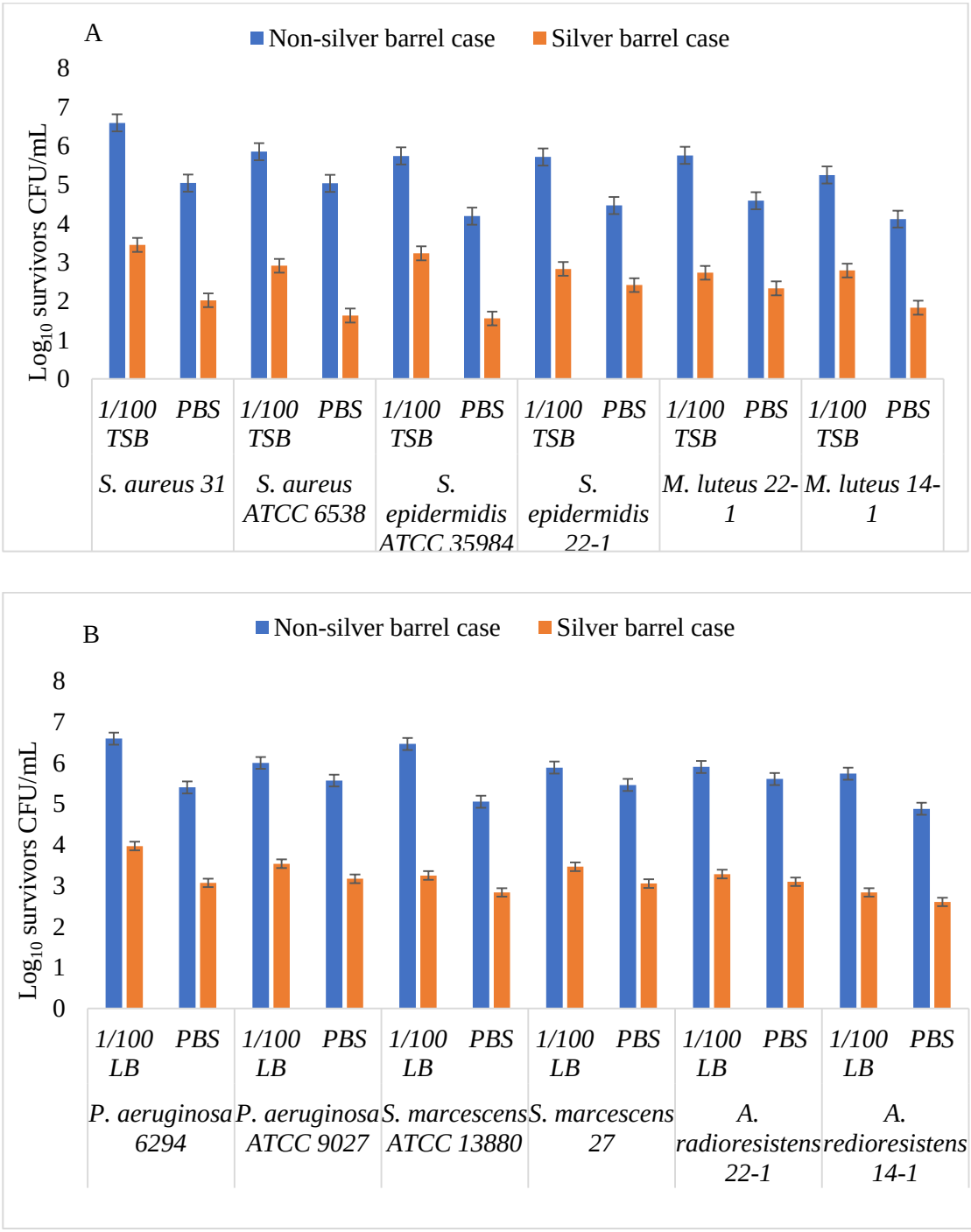


Figure 5-1: Numbers of (A) Gram positive and (B) Gram negative bacterial cells survived from silver and non-silver lens cases in different growth medium.

5.3.2 Efficacy of Synergi® disinfecting solution with or without silver lens cases

In the absence of a silver lens case, Synergi® significantly reduced the numbers of adherent Gram positive or Gram negative bacteria (p = 0.003; **Figure 5-2**). The numbers of all bacteria that could be cultured from lens cases increased as the incubation time increased, particularly from 6 to 18 hours (p = 0.002). For all bacteria, no colonies could be grown after addition of Synergi® to cells that had been allowed to adhere to cases for 6 hours. On average the reduction in bacteria numbers with the use of Synergi® on Gram positive bacteria grown for 6 hours in cases in PBS was 2.92 ± 0.3 Log_{10} CFU/mL and in 1/100 TSB was 3.34 ± 0.52 Log_{10} CFU/mL, and for Gram negative bacteria grown for 6 hours in cases in PBS was 3.49 ± 0.33 Log_{10} CFU/mL and in 1/100 LB was 3.91 ± 0.32 Log_{10} CFU/mL. For bacteria adherent to lens cases for 18 or 48 hours, there were reductions in Gram positive numbers after addition of Synergi® of 2.25 ± 0.57 or 2.25 ± 0.84 Log_{10} CFU/mL respectively for cells in PBS, and 2.94 ± 0.43 or 3.04 ± 0.91 Log_{10} CFU/mL respectively for cells in 1/100 TSB. For Gram negative bacteria there were reductions in bacteria adherent to lens cases for 18 or 48 hours after addition of Synergi® of 2.3 ± 0.38 Log_{10} CFU/mL or 2.19 ± 0.35 Log_{10} CFU/mL respectively in PBS, and 2.71 ± 0.3 Log_{10} CFU/mL or 2.18 ± 0.42 Log_{10} CFU/mL respectively in 1/100 LB. Synergi®, in the absence of a silver lens case, was most effective against *S. aureus* ATCC 6538 within the Gram positive bacteria, giving a reduction of 4.22 ± 0.2 Log_{10} CFU/mL (**Figure 5-2A**) after 48 hours in 1/100 TSB, and against *S. marcescens* ATCC 13880 within the Gram negative bacteria, given a reduction of 4.39 ± 0.2 Log_{10} CFU/mL (**Figure 5-2B**) after 6 hours exposure in 1/100 LB.

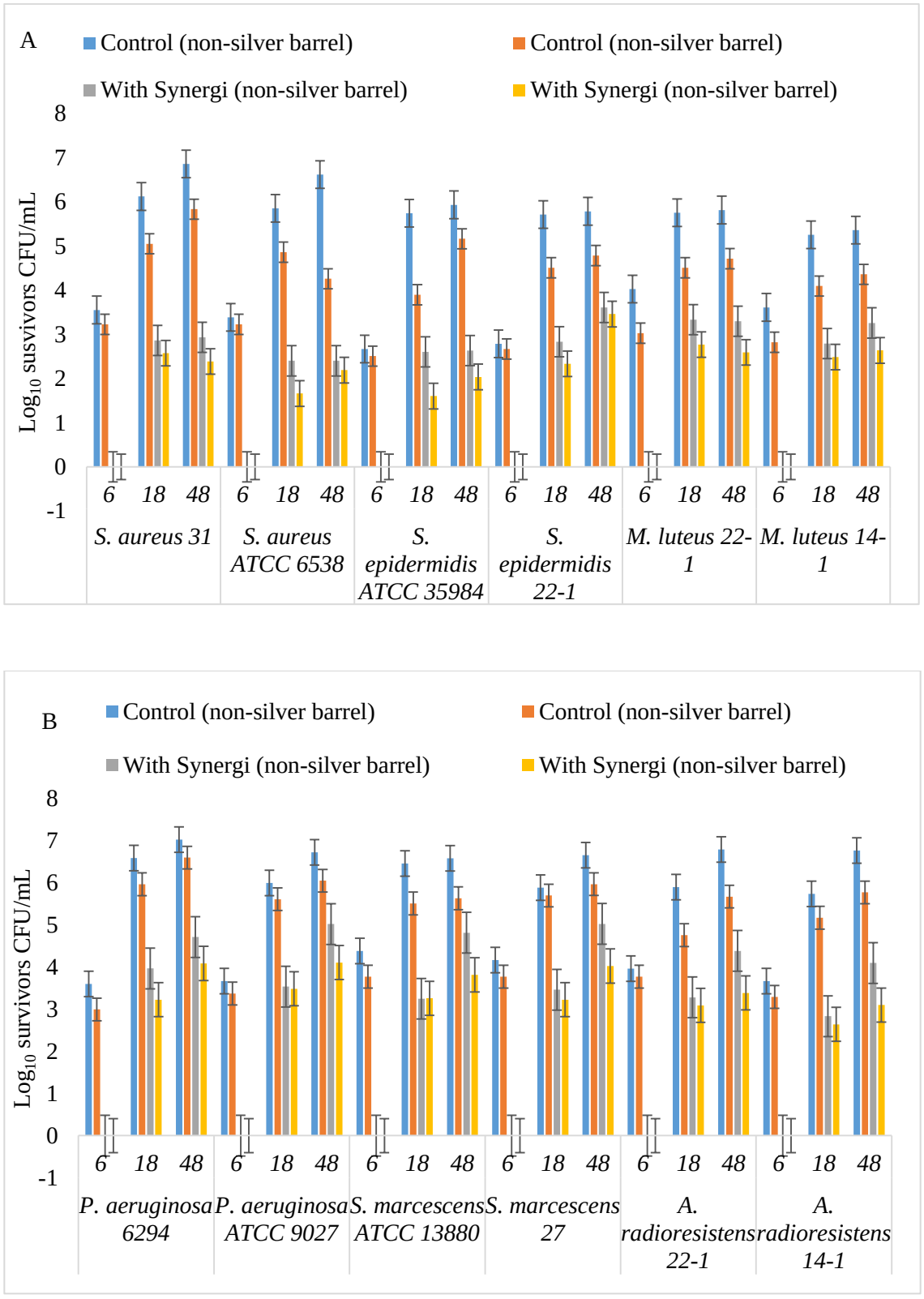


Figure 5-2: Biocidal efficacy of Synergi® disinfecting solution against (A) Gram positive bacteria and (B) Gram negative bacteria, after 6, 18 and 48 hours of bacterial adhesion in non-silver barrel lens cases with 6 hours of disinfection time.

The combination of Synergi® and silver in lens cases reduced bacterial adhesion over and above the effect of silver lens cases or Synergi® alone. Compared to silver cases alone, the addition of Synergi® significantly ($p < 0.005$) reduced the numbers of all Gram positive and Gram negative bacteria adhered to lens cases irrespective of the media in which they had been incubated or the time of adherence. For all Gram positive bacteria, addition of Synergi® with silver lens cases reduced the numbers of bacteria that could be cultured to <1 Log_{10} CFU/mL after 6 and 18 hours of adhesion to cases, compared to the silver lens cases alone. This was also the case for most of the strains of Gram negative bacteria, the exception being for both strains of *P. aeruginosa* where there were between 0.43 and 1.5 Log_{10} CFU/mL remaining after 6 and 18 hours of adhesion (**Figure 5-3**). The overall reduction in the numbers of bacteria that could be grown after addition of Synergi® to silver lens cases was significantly ($p = 0.01$) greater after 18 hours of adhesion compared to the other time points. The maximum reduction in the number of bacteria with Synergi® (for 6 hours) in silver cases for Gram positive bacteria, grown for 18 hours in PBS was 2.21 ± 0.3 Log_{10} CFU/mL against *S. aureus* 31 and in 1/100 TSB was 3.56 ± 0.52 Log_{10} CFU/mL against *M. luteus* 22-1, and for Gram negative bacteria grown for 18 hours in cases in PBS was 3.48 ± 0.33 Log_{10} CFU/mL against *S. marcescens* ATCC 13880 and in 1/100 LB was 2.14 ± 0.24 Log_{10} CFU/mL against *P. aeruginosa* ATCC 9027 (**Figure 5-3**).

Compared to the use of Synergi® without silver cases, Synergi® used with silver significantly ($p = 0.004$) reduced the numbers of all bacteria that could be cultured from cases after they had been allowed to adhere for 18 hours. For Gram positive bacteria, the use of Synergi® alone was as effective as the combination of Synergi® in silver cases after the bacteria had adhered for 6 hours. For Gram positive bacteria that

had adhered to lens cases for 48 hours, the combination of Synergi® and silver cases provided an additional 1.98 ± 0.28 Log_{10} CFU/mL reduction in bacterial numbers compared to silver lens cases efficacy alone irrespective of the media used to adhere bacteria. The maximum inhibition of 2.43 ± 0.28 Log_{10} CFU/mL against *M. luteus* 14-1 was achieved compared to the silver case alone, when bacterial cells were diluted in PBS and incubated for 48 hours. Similarly, for Gram negative bacteria adhered to lens cases for 48 hours, the combination of Synergi® and silver cases gave 1.13 ± 0.09 Log_{10} CFU/mL reduction in bacterial numbers compared to Synergi® or silver cases alone.

The individual performance of silver lens cases and Synergi® disinfecting solution was compared and Synergi® demonstrated superior efficacy in reducing bacterial adhesion compared to the silver cases alone ($p < 0.005$). The number of bacteria recovered from silver cases was 2.00 Log_{10} CFU/mL more for all bacterial strains compared to the efficacy of Synergi® disinfecting solution, regardless of the suspension media of PBS or 1/100 TSB. The exception to this was for Gram positive strains of *S. aureus* ATCC 6538, *S. epidermidis* 22-1 and *M. luteus* 22-1 and for Gram negative bacteria *P. aeruginosa* 6294 and *A. radioresistens* 22-1 after 18 hours of bacterial adhesion in PBS (**Table 5.3**). For Gram negative bacteria there was no significant difference between the biocidal efficacy of silver cases and Synergi® individually after 6 and 48 hours of bacterial adhesion (**Table 5.3**).

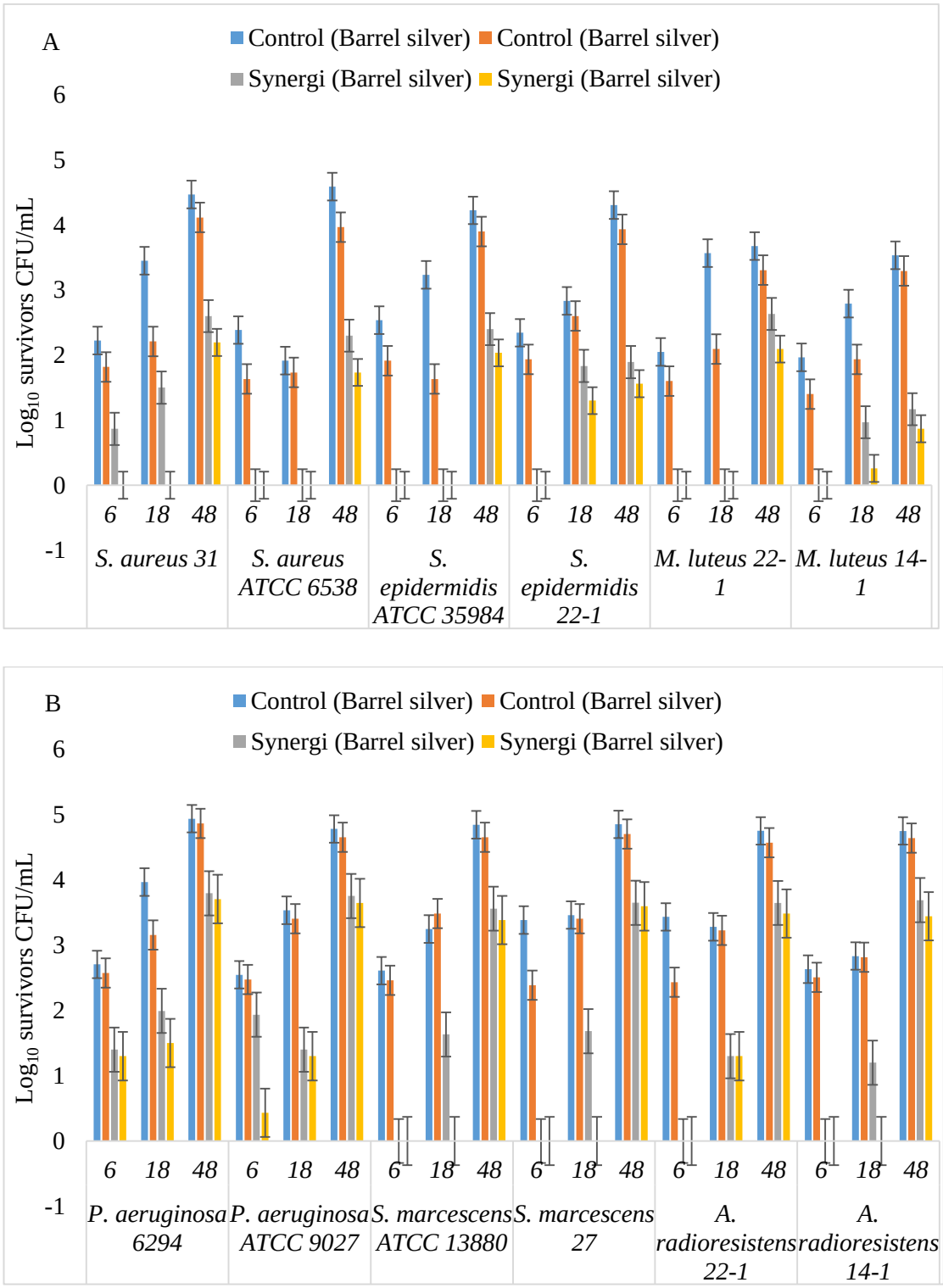


Figure 5-3: Biocidal efficacy of silver barrel lens cases in conjunction with Synergi® disinfecting solution against the test strains of (A) Gram positive and (B) Gram negative bacteria, after 6, 18 and 48 hours of bacterial adhesion formation in non-silver barrel lens cases with 6 hours of disinfection time.

Table 5.3: Difference in efficacy between silver cases along with Synergi® disinfecting solution and Synergi® efficacy alone after 6,18 and 48 hours of bacterial adhesion in PBS. Negative values indicate numbers of adhered bacteria were reduced with the combination of Synergi® plus silver lens cases compared to Synergi® alone.

		Reduction in bacterial adhesion Log_{10} CFU/mL		
	Bacterial strains	**6 hrs**	**18 hrs**	**48 hrs**
Gram positive bacteria	*S. aureus* 31	0	-2.57*	-0.2
	S. aureus ATCC 6538	0	-1.66*	-0.76*
	S. epidermidis ATCC 35984	0	-1.6*	0
	S. epidermidis 22-1	0	-1.03*	-1.9*
	M. luteus 22-1	0	-2.77*	-0.5*
	M. luteus 14-1	0	-2.23*	-1.77*
Gram negative bacteria	*P. aeruginosa* 6294	1.3*	-1.73*	-0.38
	P. aeruginosa ATCC 9027	0.43	-2.19*	-0.46
	S. marcescens ATCC 13880	0	-3.26*	-0.43
	S. marcescens 27	0	-3.23*	-0.43
	A. radioresistens 22-1	0	-1.79*	0.1
	A. radioresistens 14-1	0	-2.64*	0.34

**, represents significant inhibition ($p < 0.005$).*

5.3.3 Efficacy of Synergi® disinfecting solution with or without silver lens cases in the presence of organic soil and contact lenses

The addition of organic soil to the disinfection process resulted in a significant (p = 0.005) decrease in disinfection efficacy of ≥1.7 Log_{10} CFU/mL for all strains of Gram positive and ≥3.0 Log_{10} CFU/mL for Gram negative bacteria with or without silver lens cases after 6 hours of disinfection (**Table 5.4**). The addition of a contact lens during the disinfection process resulted in reduction in the number of viable bacteria that could be cultured from lens cases regardless of the incubation media or use of silver in cases (p = 0.005). This decrease in the number of bacteria grown in the presence of a contact lens was usually <1 Log_{10} CFU/mL, with the exception of *S. aureus* ATCC 6538, *P. aeruginosa* 6294, *P. aeruginosa* ATCC 9027 and *S. marcescens* ATCC 13880 disinfected for 10 hours in the absence of silver, or *P. aeruginosa* 6294, *P. aeruginosa* ATCC 9027, *S. marcescens* ATCC 13880 and *S. marcescens* 27 disinfected for 24 hours in the presence of silver, where the decrease in bacterial numbers was ≥1 Log_{10} CFU/mL (**Figure 5-4**). No bacteria were recovered from contact lenses for most of the strains of Gram positive bacteria, when lenses were added for 6, 10 or 24 hours during Synergi® disinfection, with the exception of *S. aureus* 31 and *S. epidermidis* 22-1 which demonstrated 1.30 Log_{10} ± 0.2 CFU/mL and 1.40 Log_{10} ± 0.1 CFU/mL (p < 0.005) bacterial adhesion in lenses respectively, after 24 hours of incubation during disinfection in non-silver cases. For Gram negative bacteria, on average by 1.50 ± 0.1 Log CFU/mL and 1.40 ± 0.1 Log CFU/mL bacterial adhesion was seen after 24 hours of Synergi® disinfection with or without silver cases respectively, but no bacterial recovery was seen when lenses were added for 6 or 10 hours during disinfection.

Overall, the combination of silver lens cases along with Synergi® was the most effective in reducing bacterial adhesion (**Figure 5-5**). The combination of silver lens cases with Synergi® disinfecting solution showed complete inhibition of bacterial adhesion against *S. aureus* ATCC 6538, *S. epidermidis* ATCC 35984, *M. luteus* 22-1 and *S. marcescens* ($p < 0.005$; **Figure 5-5**).

Table 5.4: The biocidal efficacy of Synergi® disinfecting solution in presence or absence of organic soil after 18-24 hours of bacterial adhesion in silver or non-silver lens cases. Negative values indicate the reduction in biocidal efficacy of Synergi® and silver cases in presence of organic soil in bacterial suspension.

	Reduction in biocidal efficacy of Synergi in presence of organic soil in CFU/mL	
Gram positive bacteria	**Non-silver lens cases**	**Silver lens cases**
S. aureus 31	-1.49*	-3.17*
S. aureus ATCC 6538	-2.37*	-3.17*
S. epidermidis ATCC 35984	-2.40*	-2.73*
S. epidermidis 22-1	-1.68*	-3.60*
M. luteus 22-1	-1.23*	-3.13*
M. luteus 14-1	-1.07	-2.89*
Gram negative bacteria		
P. aeruginosa 6294	-1.89*	-2.83*
P. aeruginosa ATCC 9027	-1.63*	-3.01*
S. marcescens ATCC 13880	-1.85*	-4.30*
S. marcescens 27	-1.45*	-4.18*
A. radioresistens 22-1	-1.62*	-3.33*
A. radioresistens 14-1	-1.96*	-3.33*

*, represents significant inhibition ($p < 0.005$).

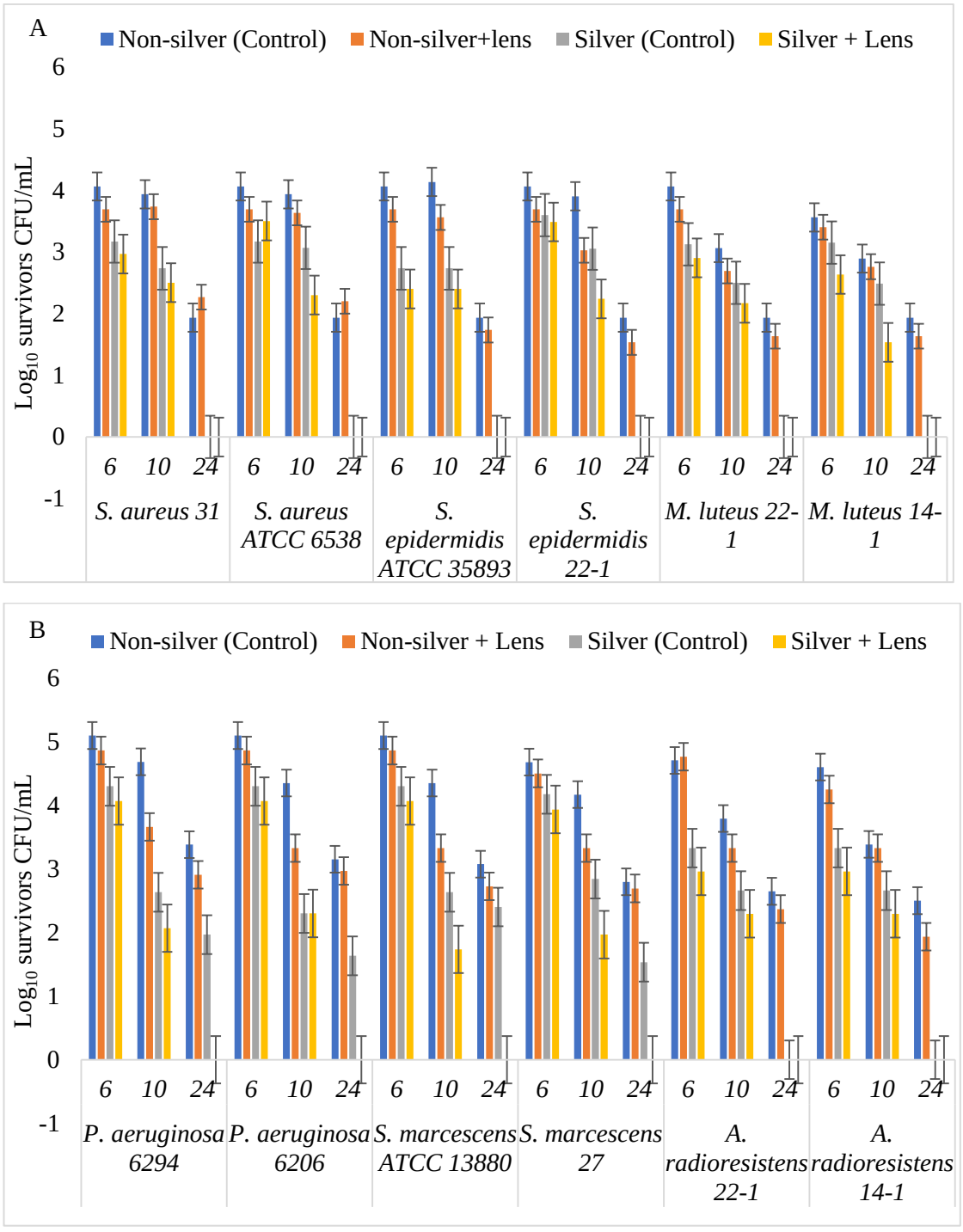


Figure 5-4: Efficacy of silver or non-silver lens cases in conjunction with Synergi® disinfecting solution against (A) Gram positive and (B) Gram negative in organic soil. Three-time points of 6 hrs, 10 hours and 24 hours represent the disinfection exposure time of Synergi® disinfecting solution.

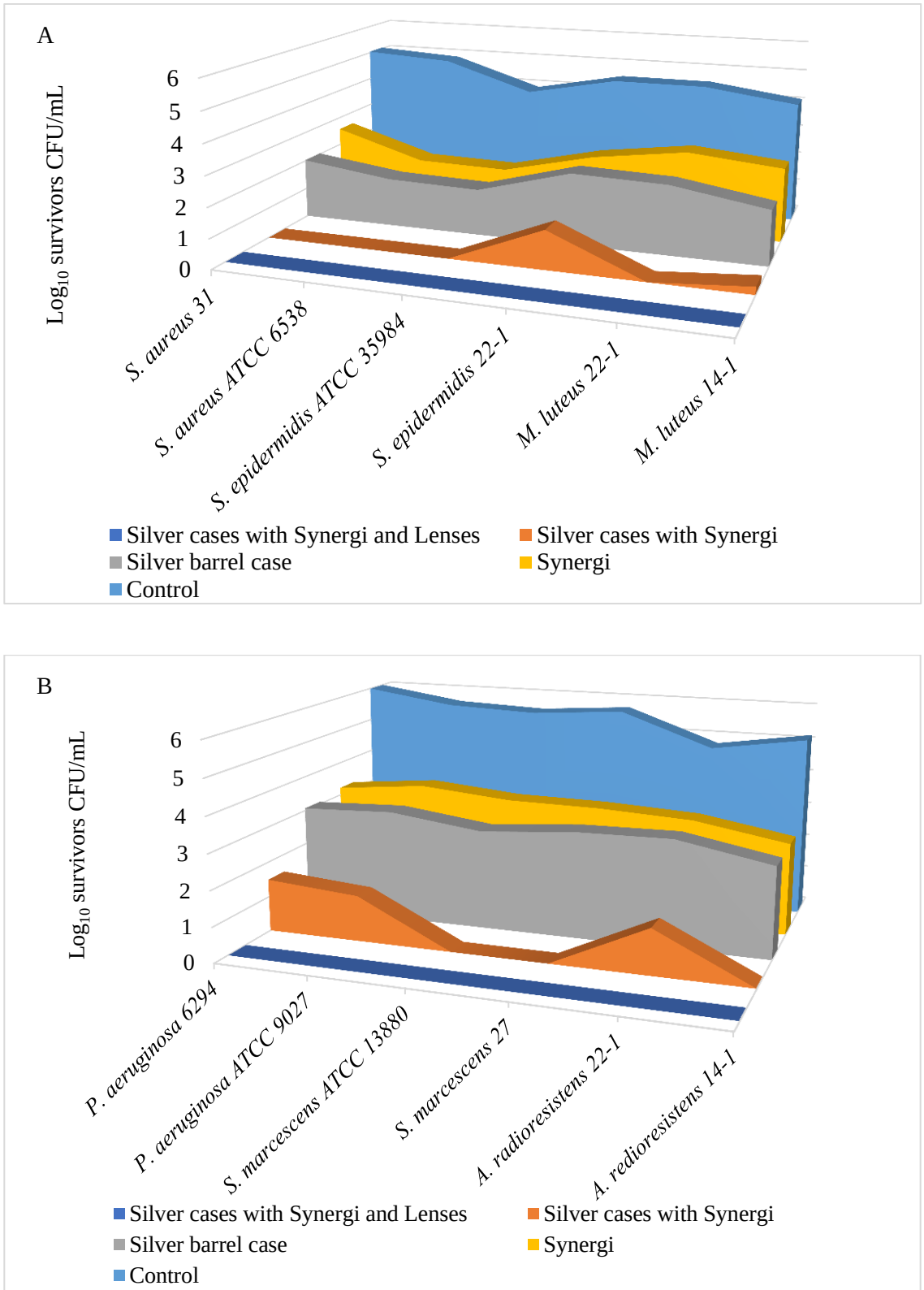


Figure 5-5: Summary of recovered bacteria of (A) Gram positive and (B) Gram negative bacteria from silver and control lens cases with different treatment options after 18-24 hours of bacterial adhesion.

5.4 DISCUSSION

Silver-impregnated barrel cases demonstrated robust activity against Gram positive and Gram negative bacteria. The greatest antimicrobial efficacy was achieved with the combined effect of silver lens cases and Synergi® disinfecting solution. The presence of contact lenses in the lens case during the disinfection resulted in a significantly greater reduction in the numbers of bacteria adhered to lens cases compared to the absence of lenses. However, the addition of organic soil increased the bacterial adhesion to lens cases and significantly decreased the disinfection efficacy of Synergi® disinfecting solution with or without silver lens cases.

For certain strains of *S. aureus*, *S. epidermidis*, *M. luteus*, *S. marcescens* and *A. radioresistens* there was complete bacterial inhibition after 6 hours of bacterial adhesion with the recommended 6 hours of Synergi® disinfection. Mowrey-Mckee *et al.* using the ISO stand alone procedure, reported that Synergi® was efficacious against the planktonic *P. aeruginosa* at the manufacturer's recommended disinfection time (6 hours) but not against *S. aureus* ATCC 6538 (Mowrey-Mckee *et al*., 2008). However, in the present study Synergi® was highly active against adherent *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 but did not show complete bacterial inhibition if bacteria had been allowed to adhere for 18 hours.

The most effective antimicrobial efficacy was achieved by the combined effect of antimicrobial silver cases and Synergi® disinfecting solution compared to the individual efficacy of either silver cases or Synergi® solution. Also, the efficacy of silver cases along with disinfecting solution was effective against higher bacterial inocula than MicroBlock™ combined with AQuify® in the previous study (Dantam *et al*., 2011). Synergi® disinfecting solution demonstrated better antibacterial efficacy

compared to the previous published data of AQuify® disinfecting solution against Gram negative bacteria *in vitro* (Tilia *et al*., 2012). This may explain the greater effect of the silver/MPDS combination found in the present study.

The presence of contact lenses reduces the antimicrobial efficacy of multipurpose disinfecting solution (Gabriel *et al*., 2018), possibly by absorbing the disinfecting solution during soaking (Clavet *et al*., 2012). However, the present study demonstrated a greater bactericidal activity in the presence of lenses, especially for *S. marcescens* ATCC 13880. The reason for this is not known but could be due to bacteria moving from the lens cases onto the lens surface as there were bacteria recovered from the lenses after disinfection. This transmission of the bacteria from lens cases to lenses has been shown previously (Vermeltfoort *et al*., 2008).

Organic soil reduced the antimicrobial activity of Synergi® disinfecting solution and silver cases. This is consistent with a previous report that used the ISO Stand Alone Test, performed in the presence of added organic soil which found reduced antimicrobial efficacy of four commercially available contact lens multipurpose disinfecting solutions with the standard bacterial suspension without the use of organic soil (Mcgrath *et al*., 2003). The use of organic soil is optional under ISO 14729 but mandatory under the Food and Drug Administration guidelines ("FDA, 2008; ISO, 2014).

The use of Synergi® disinfecting solution with silver cases for 24 hours significantly reduced the bacterial adhesion in lens cases which is consistent with previous study findings where extended exposure in silver cases with disinfecting solution showed better antimicrobial efficacy (Zhu *et al*., 2007). Additionally, the presence of hydrogen

peroxide in Synergi® disinfecting solution can be related to the better antimicrobial activity after the extended exposure, as demonstrated earlier by 3% hydrogen peroxide disinfecting solution where better disinfection efficacy was achieved for the longer disinfection time (Hiti *et al*., 2005).

The volume of the barrel lens cases was higher (10 mL *vs* 4 mL) than the commonly used flat lens cases. The addition of higher volume of Synergi® in barrel lens cases during disinfection could enhance the efficacy of Synergi® solution (Dantam *et al*., 2012). The surface area of the lens cases also can be a contributing factor in the difference between the level of microbial adhesion to flat and barrel lens cases.

The presence of oxychlorite complex in Synergi® disinfecting solution produces hypochlorite (bleach) which is antimicrobial (Falah-Tafti *et al*., 2008). A recent report demonstrated that the antimicrobial efficacy of Synergi® disinfecting solution was significantly better than other commercially available contact lens multipurpose disinfecting systems and was equivalent to a hydrogen peroxide disinfection system (AOSept® Plus: H_2O_2; CIBA) (Tilia *et al*., 2012).

In conclusion, silver-impregnated barrel lens cases exhibited broad spectrum antimicrobial efficacy with the greatest activity against Gram positive bacteria. Silver lens cases along with their cleaning and disinfecting solution enhanced the overall efficacy of the disinfection system. The use of silver cases can be an alternative to reduce the bacterial adhesion in lens cases. The remaining question is how this improved efficacy may reduce the microbial colonisation in lens cases during *in vivo* daily use. Therefore, the following chapter investigates whether silver barrel lens cases have a similar ability to control microbial colonisation *in vivo* (Chapter 6).

Chapter 6:
In vivo Antimicrobial Efficacy of Silver Lens Cases

Acknowledgement:

Sauflon Pharmaceuticals provided the silver impregnated contact lens storage cases and Synergi solution during the PhD work.

Chapter 6 In vivo Antimicrobial Efficacy of Silver Lens Cases

6.1 INTRODUCTION

Recently, silver-impregnated barrel lens cases have been developed, Chapter 5 in this thesis explored the *in vitro* antimicrobial efficacy of silver-impregnated barrel lens cases against a wide range of clinical and standard strains of Gram positive and Gram negative bacteria. Chapter 5 demonstrated a consistent performance of silver-impregnated barrel lens cases against Gram positive and Gram negative bacteria. However, it is important to determine whether the efficacy exhibited by this storage cases *in vitro* is reflected *in vivo*. Therefore, the present study aims to examine the rate and level of contamination in silver-impregnated barrel lens cases in conjunction with contact lens cleaning and disinfecting solution *in vivo*.

6.2 MATERIALS AND METHODS

6.2.1 Contact lens storage cases, contact lenses and lens care products

Silver-impregnated storage barrel cases (Sauflon Pharmaceuticals Ltd. London, UK) and control non-silver barrel cases (Sauflon Pharmaceuticals Ltd.) were tested in conjunction with Synergi® cleaning and disinfecting solution (Sauflon Pharmaceuticals Ltd.). The Synergi® disinfecting system contains a oxychlorite complex (Oxipol™; sodium chloride and hydrogen peroxide) as the disinfecting component, with hydroxypropyl methylcellulose (HPMC) as a viscosity enhancing agent. During the clinical trial participants were allowed to use their habitual frequent replacement daily soft contact lenses (either hydrogel or silicone hydrogel materials).

6.2.2 Study design

A prospective, single centre, double-blind, crossover study was conducted to evaluate the rate and level of microbial contamination of silver and control lens cases along with Synergi® solution over a two-month period.

6.2.2.1 Ethics approval

This study was approved by the University of New South Wales (UNSW) Human Research Ethics Committee (approval ref # HC16961) and all procedures were conducted in accordance with the tenets of the Declaration of Helsinki 1975 as amended in 2000 including local regulator as applicable, such as Therapeutic Goods Administration, Australia (TGA). The clinical trial was conducted under the clinical trial notification (CTN) scheme of the Therapeutic Goods (Medical Devices) Regulations 2000 (Appendix D). The clinical trial was registered with the Australian New Zealand Clinical Trial Registry (ANZCTR; trial ID: ACTRN 12617001607369).

6.2.2.2 Sample size calculation

Assuming a lens case contamination rate of 71% from previous studies (Dantam *et al*., 2011) and the expectation that silver barrel lens cases would demonstrate a reduction of 20% (71% *vs* 51%) in case contamination, a sample size of 51 was calculated using a 5% level of significance with 80% power and assuming a 20% drop out rate. The sample size was estimated using the online sample size calculator tool (http://stat.ubc.ca/~rollin/stats/ssize/index.html). Due to the crossover study design each participant served as their own control, permitting paired statistical testing to be used to evaluate differences.

6.2.2.3 Key inclusion criteria

A total 51 participants over 18 years of age were enrolled. Participants had normal ocular signs, with no systemic or ocular contraindications to contact lens wear and with or without previous lens wear experience were recruited. Recruitment occurred by means of either email or from the database of the School of Optometry and Vision Science, UNSW. Other specific inclusion criteria are listed below:

- Be able to read and comprehend English and give informed consent as demonstrated by signing a record of participants informed consent;
- Have ocular health findings considered "normal" and which would not prevent the participant from safely wearing contact lenses;
- Be existing frequent replacement contact lens wearer (hydrogel or silicone hydrogel) with or without previous lens wear experience who are willing to wear contact lenses on a daily basis for a minimum of 4 days per week (on average) over the course of the study;
- Be willing to use the study-prescribed contact lens case and disinfecting solution for the duration of the study.

6.2.2.4 Key exclusion criteria:

Participants enrolled in the trial must NOT:

- Use daily disposable or rigid gas permeable contact lens (including orthokeratology);
- Have history of skin allergy towards any metal or chemical components in particularly with iodine sensitivity were excluded from the study enrolment;

- Have any active corneal infection (by physical investigation), past ocular disease or systemic disease (by history taking) such as diabetes, Graves disease, and auto-immune diseases such as ankylosing spondylitis, multiple sclerosis, Sjögren syndrome and systemic lupus erythematosus that would affect wearing of contact lenses or may interfere with the ocular surface properties;
- Use or need use of any systemic or topical medications which may alter normal ocular findings/are known to affect a participant's ocular health/physiology or contact lens performance either in an adverse manner or risk providing a false positive;
- Have undergone eye surgery within 12 weeks immediately prior to enrolment for this trial;
- Have contraindications to contact lens wear;
- Be currently enrolled in another clinical trial.

6.2.2.5 Masking procedure

Both the participants and the investigator were masked to the type of contact lens cases (silver or non-silver) dispensed during the clinical trial. The investigator was unmasked at the end for the data analysis of the trial.

6.2.2.6 Clinical trial randomization

A simple randomisation table was created by using the online randomization tool as per http://www.randomization.com. A randomization list was generated, and an unmasked volunteer administered for help. The silver or non-silver barrel cases were allocated based on concealed envelopes marked with the participants identification

number and the visit number. Participants received either the silver or control case at their first or second visits based on the randomization scheme.

6.2.3 Participants enrolment and study instructions:

Participants who provided written informed consent (Appendix B2) were dispensed with silver or control cases (each type for one month) along with Synergi® solution to use with their habitual daily wear soft contact lenses over a two-month period. Participants were advised to return their used lens case and the solution bottle at the end of each month and the other type of lens case was delivered with a new bottle of solution. **Figure 6-1** represents the sequence of events carried out during the recruitment and the course of the clinical trial. There was no washout period before the two treatments.

Participants were instructed to wear their contact lenses for a minimum of four days per week for an average of six hours a day during the study period and to replace the lenses according to the manufacturers recommended schedule. Upon daily removal of the lens from the eye, participants were advised to place these lenses directly into the appropriate case compartment and close the basket. The participants then filled the lens case to the indicated with Synergi® solution. After this the lens cases were recapped and left for a minimum of four to six hours (the manufacturer's recommended minimum disinfection time) for disinfection. Upon removal of the contact lenses from the lens case, participants were instructed to rinse their lenses with the Synergi® solution prior to inserting lenses into their eyes. The participants were advised to read the manufacturers hygiene instructions provided with the disinfecting solution (Appendix F).

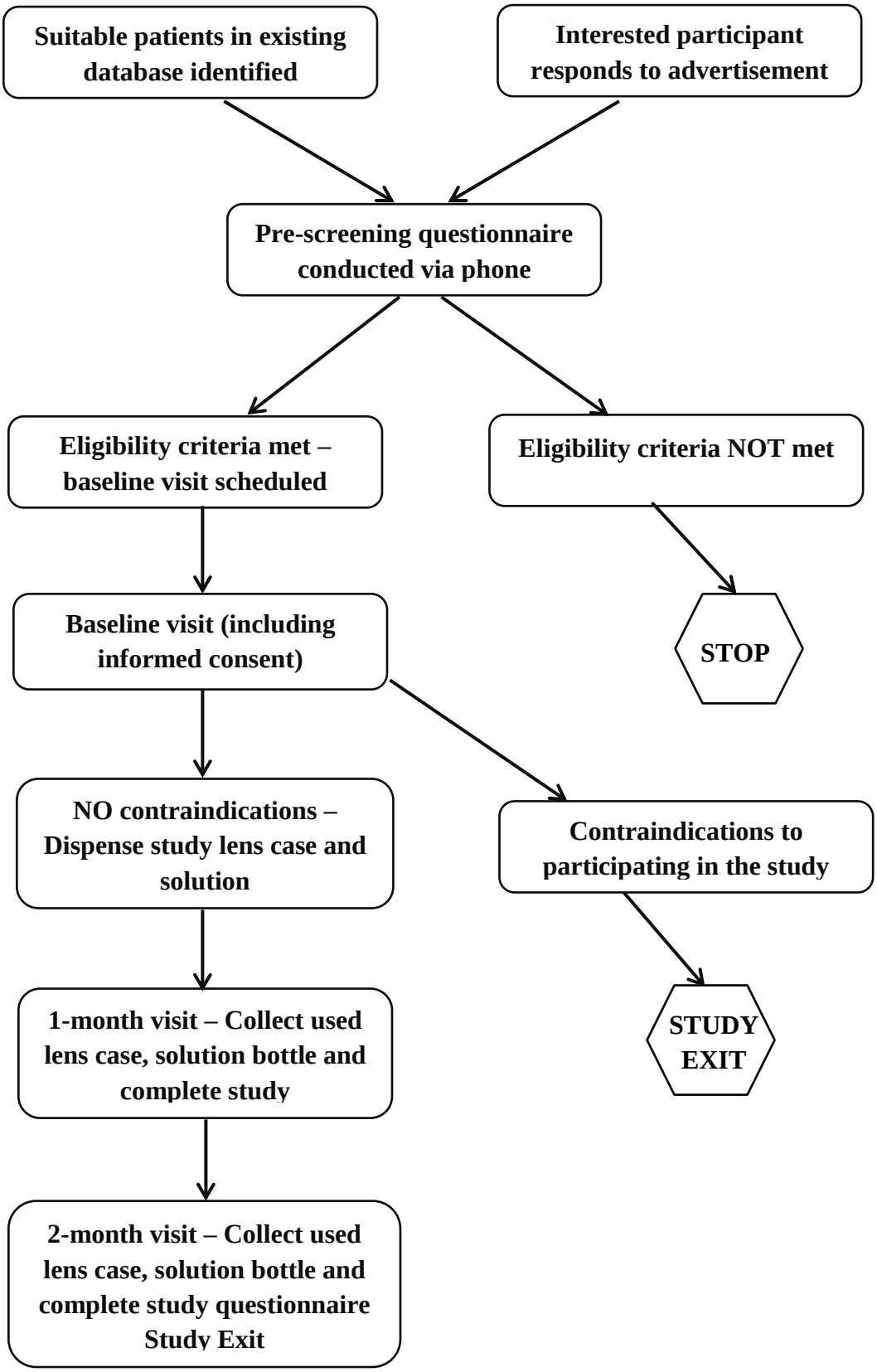


Figure 6-1: Study methodology for silver and non-silver barrel lens case trial.

6.2.4 Clinical examination:

An assessment of lens fitting, and anterior eye health examination was conducted at each visit. Anterior ocular health (bulbar and limbal conjunctival redness, extent of corneal and conjunctival staining, and palpebral redness and roughness) was graded using the CCLRU grading scale (Terry *et al*., 1993) using a Zeiss SL-120 biomicroscope (Carl Zeiss Meditech, Jena, Germany). The subjective ratings and ocular health variables measured at baseline were considered to represent the clinical performance of the participant's habitual lens care product and assessments conducted at 1st month and 2nd month were representative of the silver and Synergi® disinfecting system with the participant's habitual contact lenses. Lens case and solution bottles were collected at the first and second month visits (**Figure 6-1**). The clinical trial was conducted under a similar protocols to those reported previously (Chapter 2); (Carnt *et al*., 2009; Willcox *et al*., 2010) with the exception that the participants used their habitual contact lenses. The schedule of visits and the data requirements for clinical techniques of each visit including the baseline visit are shown in **Table 6.1**.

Table 6.1: Clinical techniques and assessment of variables of each visit.

Procedures	***Visit 1*** **Baseline**	***Visit 2*** **(1 month from baseline)**	***Visit 3/Study Exit*** **(2 month from baseline)**	**Unscheduled / Adverse events**
Visit Window	N/A	± 7 days	± 7 days	**N/A**
Informed consent	Y	N	N	**N**
Meet inclusion/exclusion criteria	Y	N	N	**N**
Ocular and medical history, medications, demographics	Y (I)	N	N	**N**
Updated history, symptoms and problems	N	Y (I)	Y (I)	**Y (I)**
Vision tests (Visual acuity)	Y (M)	Y (M)	Y (M)	**Y (M)**
Slit-lamp bio microscopy: Lens fitting, anterior ocular health including evaluation of cornea & conjunctiva with fluorescein	Y (M)	Y (M)	Y (M)	**Y (M)**
Questionnaires and Rating scales	N	Y (M)	Y (M)	*****
Lens Case and Solution Bottle Returns	N	Y (I)	Y (I)	**N**
Return Unused Study Lens Care Product	N	N	Y (I)	*****
Adverse Event Data	**(Y)****	**(Y)****	**(Y)****	**Y**

N/A = not applicable; Y = Yes; information recorded; N = No; not recorded; I = Interview; M = in clinic measurement or observation.

* At optometrist's discretion

** If adverse event detected at time of visit

6.2.5 Compliance score calculation:

At each visit, compliance with the minimum lens wear requirements and solution regimens were evaluated by a self-administered questionnaire (Appendix C). The compliance score was calculated from the factors which were significantly associated with microbial contamination of lens cases (Wu *et al*., 2015; Wu *et al*., 2010a). The compliance score was calculated using the number of positive behaviour responses divided by the total number of behaviours analysed (**Table 6.2)**. A correct response was identified as "yes" or "no", depending on the question in the compliance questionnaire. Actual compliance was defined based on a compliance score and grouped into one of three categories which was adopted from the study by Morgan *et al.,* with modification (Morgan, 2007). A patient was considered to have good compliance when receiving a 90% (demonstrating 11 of 12 correct behaviours) or better pass rate. Average compliance was defined as 70% pass rate (demonstrating 9 of 12 correct behaviours), and poor compliance was defined as 60% or worse pass rate (demonstrating 8 or fewer correct behaviours of 12).

Table 6.2: Compliance questionnaire related to contact lens wear.

Factors considered in compliance questionnaire†
Compliant practices*
1. Washing hands before handling lenses with soap
2. Drying hands prior to handling lenses
3. Rubbing of contact lens
4. Rinsing of contact lens
5. Rubbing of contact lens cases
6. Rinsing of contact lens cases
7. Air-drying of lens cases
8. Using fresh solution
9. Filling up to the mark line on lens case
10. Using goggles during water sports
Non-compliant practices*
11. Topping off (adding) solution to lens case
12. Wearing contact lenses while showering/water sports

*, for complaint practices "*Yes*" was considered as positive compliance and "*No*" and "*Unsure*" were considered as negative compliance behaviour. Similarly, for non-complaint practices "*No*" was considered as positive compliance and "*Yes*" and "*Unsure*" were considered as negative compliance behaviour.

†, full questionnaire is given in "Appendix C".

6.2.6 Microbial culture collection:

Lens cases were sent to the microbiology laboratory within one hour of collection or stored at 4ºC if the cases could not be processed within that time – and then processed at earliest opportunity. Lens cases were swabbed with a sterile cotton swab premoistened with phosphate buffer saline (PBS; pH 7.4 NaCl 8 g 1^{-1}, KCl 0.2 g 1^{-1}, Na_2HPO_4 1.15 g 1^{-1}, KH_2PO_4 0.2 g 1^{-1} pH 7.2). One swab was used for both the case, basket and lid.

The swab was placed in 2 mL of PBS and vortexed at 700 *g* for 10-20 seconds, then 400µL of the PBS was inoculated onto each of three chocolate blood agar plates (Thermo Fisher Scientific, Australia) and one Sabouraud's dextrose agar plate (Thermo Fisher Scientific, Australia). The chocolate blood agar plates were incubated at 37ºC for 24, 48 hours and 96 hours under aerobic, microaerophilic and anaerobic conditions, respectively and the Sabouraud's dextrose agar plate was incubated at room temperature for 1 week for fungal recovery. A total colony count was performed to determine the number of microbes on each plate. Preliminary identification involved examination of colony morphology followed by microscopy and Gram staining (Remel™ Gram staining kit; Thermo Fisher Scientific, Australia) for bacteria. Unique colonies were preserved in 50% glycerol at -80ºC for the further identification of bacteria using biochemical techniques.

6.2.7 Bacterial identification protocol:

6.2.7.1 Gram positive cocci:

Initially, the catalase test with 3% hydrogen peroxide (Sigma-Aldrich, Castle Hill, Australia) was performed for Gram-positive cocci. Catalase-negative cocci were recorded as *Streptococcus* spp. Streptococci were further identified by their

microscopic morphology and sensitivity tests using 10µg vancomycin (Thermo Fisher Scientific, Australia) (Facklam & Elliott, 1995) and optochin (Thermo Fisher Scientific, Australia) (Gardam & Miller, 1998) to differentiate *Streptococcus pneumoniae* from other species. Catalase-positive cocci were identified to the genus level using biochemical tests (Leitch, Harmis, Corrigan, & Willcox, 1998), resistance to furazolidone (Thermo Fisher Scientific, Australia) on sensitest agar plates (ISO-Sensitest Agar; Oxoid Biochemical Identification System) followed by growth on mannitol salt agar (Thermo Fisher Scientific, Australia); DNase Test (DNase Agar; Oxoid, Biochemical Identification System, Australia); pyrrolidonyl arylamidase (Oxoid, Biochemical Identification System, Australia); coagulase test (Staphylase test kit-DR595A, Oxoid Biochemical Identification System, Australia) as shown in **Figure 6-2**.

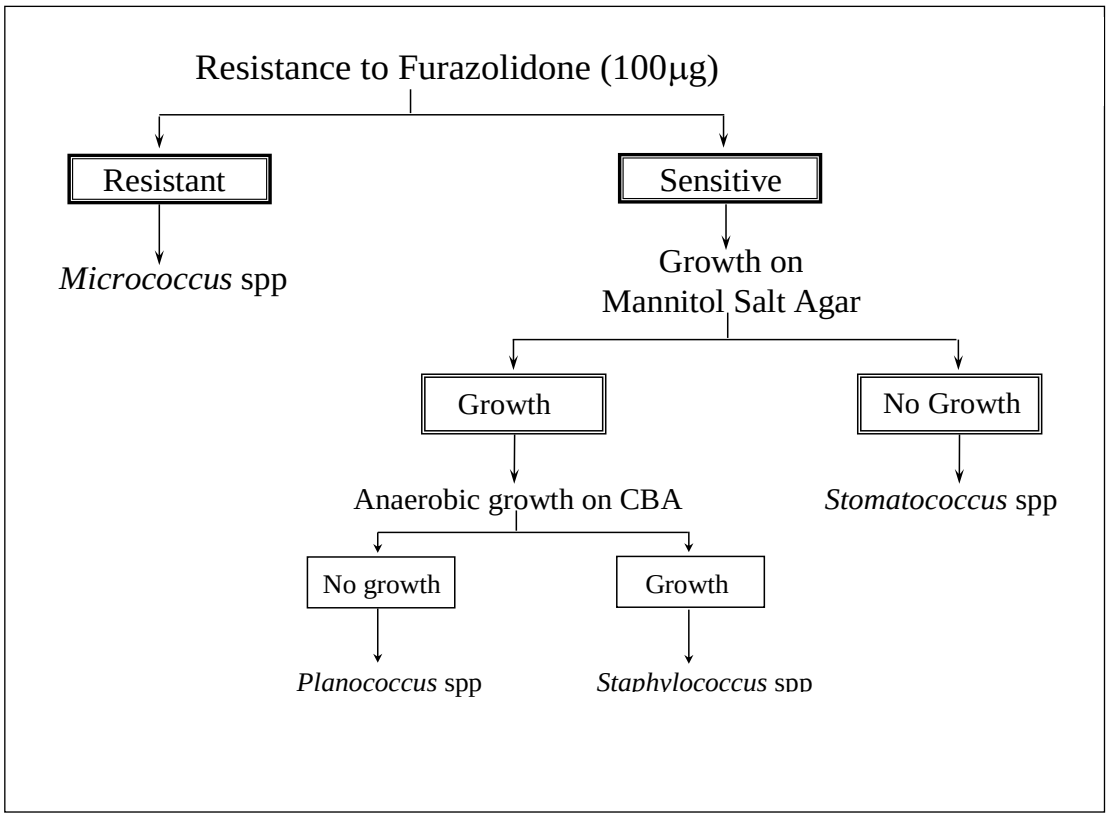


Figure 6-2: Flow chart for identification of catalase-positive cocci.

6.2.7.2 Gram positive and Gram negative rods:

Gram positive rods were further identified by determining the presence of endospores after growth on nutrient agar plates (Thermo Fisher Scientific, Australia) and staining a single colony on a microscope slide (Leica Microsystems Pty Ltd. Australia) with a 5% solution of Malachite green (Bacto-Laboratories Pty Ltd, Australia) and using bright-field light microscopy with the 100x oil-immersion objective to discern colours (Balows, Hausler, Hermann, Isenberg, & Shadomy, 1991; Murray, Baron, Pealler, Tenover, & Yolken, 1995); endospores appeared as minty green inclusions in bacterial cells. Further identification steps are outlined in **Figure 6-3**. Gram-negative rods were divided into oxidase positive and oxidase negative and were further identified using commercially available API kits (API; Biomerieux, France). For oxidase positive strains API 20NE was used and for oxidase negative strains API20E or API Rapid 32E were used.

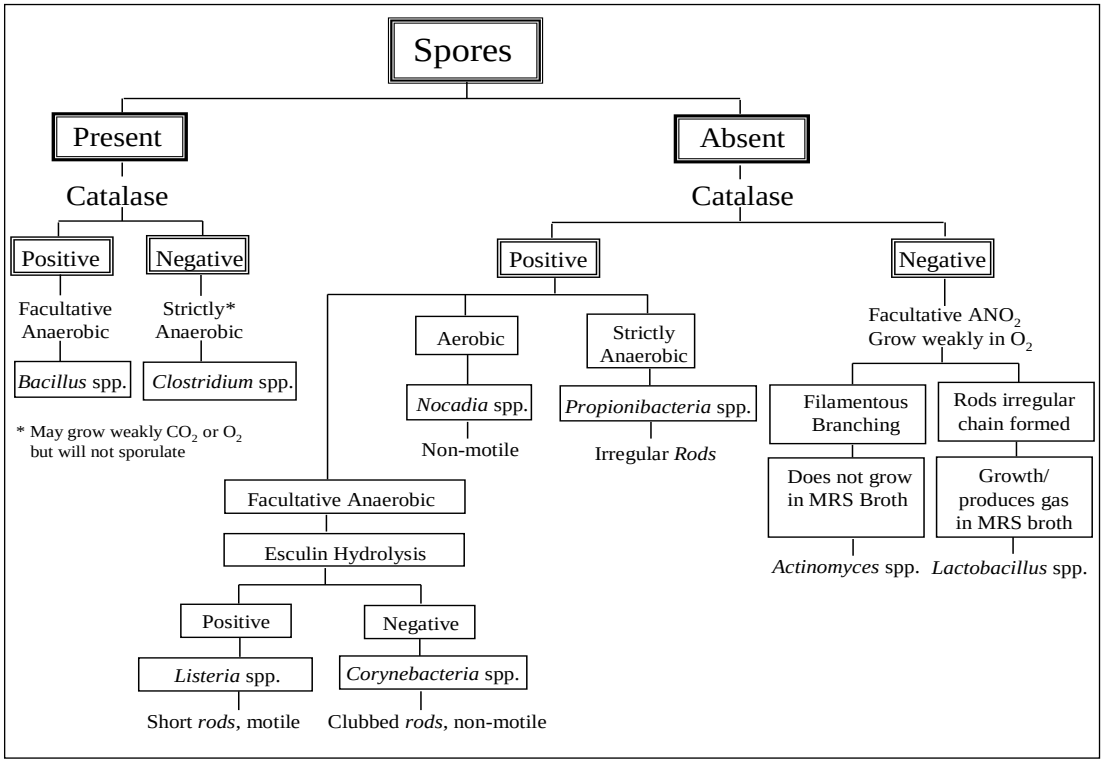


Figure 6-3: Flow chart for identification of Gram positive rods.

6.2.8 Fungal identification

Positive cultures on Sabouraud's dextrose agar plates were identified by macroscopic morphology to differentiate between yeast (lack of hyphae) and filamentous fungi.

6.3 STATISTICAL ANALYSIS:

The total number of viable organisms was recorded as colony forming unit (CFU) per lens well and converted into Log10 CFU/mL. Data analysis was performed using Microsoft Excel 2010 and Statistical Package for Social Science for Windows version 20.0 (SPSS, Inc, Chicago, IL). Levels of microbial contamination (CFU) of cases were log transformed for the data analysis. A linear mixed model was used to compare the levels of contamination between silver and control cases and also for different storage conditions after adjusting for any intra-subject correlations and contact lens wearing schedule. The Mann-Whitney U test was used to compare the percentage of Gram positive and Gram negative bacteria recovered.

A linear mixed model was used to compare the ocular signs of subjects and the data of the compliance questionnaire to determine any association with microbial contamination. Chi-Squared tests were used to compare subjective symptoms and to determine associations between symptoms and microbial contamination of lens cases.

6.4 RESULTS:

6.4.1 Demographics:

A total of 51 participants (36 females and 15 males) with an average age of 29 ± 13 years (range 18 to 62 years inclusive) were enrolled. Two participants did not return contact lens case at their final visit, and so the 2nd month data of these participants were excluded from the data analysis. Ninety-six percent (47/49) of the participants wore single vision contact lenses either to correct myopia or hyperopia, and astigmatism and 4% of participants (2/49) used multifocal contact lenses for presbyopia correction. The average lens wearing time was 10 ± 3 hours in a day (ranged from 5 to 17 hours) with no difference between test and control groups and 63% (31/49) of participants wore contact lenses for more than 4 days a week and the rest at least 4 days a week. Fourteen percent (7/49) of the participants had worn contact lenses for 1-2 years prior to the study enrolment, 53% (26/49) for more than 3 years and 22% (11/49) had worn contact lenses for more than 10 years.

Thirty-six (71%) participants wore silicone hydrogel contact lenses with the most common being senofilcon A (13 participants) followed by comfilcon A (11 participants) and lotrafilcon B (6 participants) **Table 6.3**. Thirty-one (61%) of the participants used monthly disposable lenses, 13 (25%) bi-weekly disposable lenses. The remainder of the subjects used yearly or half-yearly disposable contact lenses. One participant routinely used a peroxide disinfection system prior to the study enrolment, while the remainder used a variety of multipurpose solutions. The details of the contact lens material and the solution combination used habitually by participants prior to the study are provided in **Table 6.3** and **Table 6.4**.

Table 6.3: Habitual contact lens materials and the manufacturing companies of the contact lenses used by participants during the study.

Contact lens Material (Manufacture)	No. of subjects
Silicone hydrogels	
Senofilcon A (Johnson & Johnson Vision Care, Inc., Jacksonville, FL)	13 (25%)
Comfilcon A (Cooper Vision, Pleasanton, CA)	11 (22%)
Lotrafilcon B (Alcon Laboratories Inc., Texas, USA)	6 (12%)
Filcon II 3 (Sauflon, Twickenham London)	2 (4%)
Balafilcon A (Bausch & Lomb, New York, USA)	1 (2%)
Balafilcon B (Bausch & Lomb, New York, USA)	1 (2%)
Fanfilcon A (Cooper Vision, Pleasanton, CA)	1 (2%)
Hydrogels	
Etafilcon A (Johnson & Johnson Vision Care, Inc., Jacksonville, FL)	4 (8%)
Polymacon (Bausch & Lomb, New York, USA)	2 (4%)
Omafilcon B (Cooper Vision, Pleasanton, CA)	1 (2%)
Ocufilcon D (Cooper Vision, Pleasanton, CA)	1 (2%)
Methafilcon A (Cooper Vision, Pleasanton, CA)	1 (2%)
Alphafilcon A (Bausch & Lomb, New York, USA)	1 (2%)

Table 6.4: Habitual disinfecting solutions used by participants before enrolment in the study.

Disinfecting solution	Manufacturing company	No. of subject's N (%)
OPTI-FREE® Puremoist® (Sodium citrate, sodium chloride, boric acid, aminomethyl propanol, sorbitol, disodium EDTA, Tetronic 1304 and HydraGlyde Moisture Matrix - EOBO41, polyxylethllene-polyxybutylene with Polyquad 0.001% and Aldox 0.0006% preservatives)	Alcon Laboratories Inc.	17 (33%)
ReNu® MultiPlus® (Dymed-1 0.0001% (Polyaminopropyl biguanide; PAPB), Poloxamine, Boric acid, Sodium chloride EDTA, Sodium borate Hydranate1 (hydroxyalkylphosphonate)	Bausch & Lomb	11 (22%)
Biotrue® (Polyaminopropyl biguanide; PAPB 0.00013%, 0.0001% polyquaternium-1, Hyaluronan, sulfobetaine, poloxamine, boric acid, sodium borate, edetate disodium, sodium chloride)	Bausch & Lomb	5 (10%)
OPTI-FREE® RepleniSH® (Polyquad (polyquaternium-1) 0.001%; Aldox (myristamidopropyl dimethylamine) 0.0005%)	Alcon Laboratories Inc.	2 (4%)
Complete MoisturePlus™ (Polyhexamthylene biguanide, 0.0001%, Hydroxypropyl methylcellulose, propylene glycol, phosphate, and taurine, Poloxamer 237, edetate disodium, sodium chloride, potassium chloride, and purified water)	Abbott Medical Optics.	1 (2%)
OPTI-FREE® Express® (Polyquad (polyquaternium-1) 0.001%; Aldox (myristamidopropyl dimethylamine) 0.0005%, Tetronic, sodium chloride, edetate disodium, boric acid, amino methyl propanol, citrate)	Alcon Laboratories Inc.	1 (2%)
AOSept (Hydrogen peroxide 3%, sodium chloride 0.79%, stabilized with phosphoric acid, a phosphate buffered system, PLURONIC† 17R4 (a cleaning agent), and HydraGlyde* Moisture Matrix (EOBO-21*-polyoxyethylene-polyoxybutylene)	CIBA Vision	1 (2%)
LensCare (PHMB 0.0002%, EDTA 0.01%, HEC, Poloxamer 0.18%, polyquaternium-II 0.004%)	Lens Care	1 (2%)
Hydron (Biguanide 0.0025mg/mL, Disodium-EDTA 1.0mg/mL, Sodium Borate, Sodium Chloride)	Hydron Ltd.	1 (2%)
Reclens (Sodium Chloride, Potassium Dihydrogen Phosphate and Disodium Hydrogen Phosphate, preserved with EDTA and Polyaminopropyl Biguanide equivalent to 9g/L Sodium Chloride)	Aaxis Pacific Healthcare	1 (2%)
Unknown	_	7 (14%)

6.4.2 Ocular sign and symptoms (objective assessment):

Ocular variables were measured for both eyes, but as there were no differences between the eyes upon statistical analysis, data for the left eye are reported. No significant differences were noticed between the eyes of participants using silver and non-silver barrel lens cases in bulbar and limbal conjunctival redness, extent of conjunctival staining and palpebral redness and roughness ($p > 0.05$; **Table 6.5**). However, there was a significant difference in conjunctival staining and palpebral redness between the participants' habitual lens care product at baseline compared to Synergi® disinfecting solution in combination with silver lens cases at the one-month or two-month visits ($p = 0.03$; **Table 6.5**).

6.4.3 Survey questionnaires:

No significant differences were found in subjective comfort or vision between the use of the participants' habitual lens care product at baseline, and Synergi® disinfecting solution with or without silver lens cases **Table 6.5**. However, there was a trend for participants to report an improvement in itchiness but slightly reduced end day comfort with Synergi® compared to their habitual lens care product ($p < 0.05$; **Table 6.5**). The maximum comfortable contact lens wearing time with Synergi® disinfecting system was 7.7 ± 1.2 hours.

Table 6.5: Objective and subjective variables recorded at each visit to the clinic.

Ocular health[a]	**Baseline**	**Silver**	**Control**[b]	**P value**[c]
Bulbar redness_Nasal	1.40 ± 0.7	1.33 ± 0.7	1.33 ± 0.7	
Bulbar redness_Temporal	1.21 ± 0.7	1.27 ± 0.6	1.27 ± 0.6	
Bulbar redness_Superior	1.33 ± 0.7	1.31 ± 0.6	1.30 ± 0.6	
Bulbar redness_Inferior	1.17 ± 0.7	1.17 ± 0.6	1.18 ± 0.6	
Limbal redness_Nasal	0.69 ± 0.5	0.78 ± 0.6	0.77 ± 0.6	0.07
Limbal redness_Temporal	0.73 ± 0.5	0.69 ± 0.6	0.70 ± 0.6	
Limbal redness_Superior	0.45 ± 0.5	0.56 ± 0.5	0.55 ± 0.5	
Limbal redness_Inferior	0.49 ± 0.5	0.52 ± 0.6	0.51 ± 0.6	
Upper palpebral roughness	0.79 ± 0.8	0.93 ± 0.7	0.92 ± 0.7	
Conjunctival staining	***0.92 ± 0.9***	*1.14 ± 0.7*	*1.15 ± 0.6*	***0.03***
Upper palpebral redness	***1.04 ± 0.8***	*1.23 ± 0.6*	*1.21 ± 0.6*	
Comfort Survey _ Subjective rating (0 - 100)	**Baseline**	**Silver**	**Control**	
Comfort _ insertion	86 ± 18	88 ± 18	88 ± 18	
Vision _ insertion	87 ± 16	91 ± 17	91 ± 17	
Burning/stinging _ insertion	91 ± 21	87 ± 21	87 ± 21	
Discomfort _ during lens wear	75 ± 22	77 ± 25	77 ± 25	0.09
Dryness _ during lens wear	85 ± 23	86 ± 22	87 ± 22	
Comfort _ end of day	86 ± 26	75 ± 28	76 ± 28	
Vision _ end of day	86 ± 25	86 ± 22	87 ± 22	
Closing the eye _ end of the day	79 ± 23	83 ± 25	83 ± 25	
Itchiness _ insertion	***72 ± 14***	94 ± 14	95 ± 14	***0.05***

a, no corneal staining was observed;
b, no significant differences between silver and control lens cases;
c, italics indicates trend ($p < 0.05$) in difference between baseline visit.

6.4.4 Contact lens case hygiene practices:

Total compliance scores were not associated with the frequency and level of microbial contamination of storage cases and no difference between male and female participants, was found irrespective of the types of lens cases used. Overall, only 2% of participants demonstrated "good compliance", 12% "average compliance" and 78% "poor compliance" (**Figure 6-4)**. Ninety-five percent of the participant's reported having washed their hands prior to handing lenses, 85% of them washed with soap and 11% of them washed without soap, the remaining participants were unsure whether they have used soap or not. Sixty-nine percent of lens wearers reported rubbing and rinsing their contact lenses and 10% did not rub and rinse their contact lenses after lens removal. Approximately, 87% of participant's always disinfected and stored contact lenses with disinfecting solution whilst 8% reported occasional use of disinfecting solution. After the storage period, 28% did nothing with lenses before inserting them into their eyes, while 43% rubbed and rinsed lenses before lens insertion and 15% only rinsed contact lenses with disinfection solution (**Table 6.6)**.

Eighty-seven percent of participants usually disinfected their contact lens cases and 31% filled up the solution in the lens cases up to the mark on the lens cases. During lens cases cleaning, 37% participants reported rubbing their contact lens storage cases after lens removal with the fresh disinfecting solution and 34% of participants reused the solution remaining in the lens cases. Sixty-nine percent of participants reported airdrying their lens cases after rinsing and the rest stored their lens cases without air drying. Lens cases were more frequently stored in the bathroom (58%) compared to bedroom or in kitchen ($p < 0.02$; **Table 6.6**). Ten participants recalled that in the last

month they had used water to clean their lens cases before the enrolment in the study and one participants recalled using water during the clinical trial.

Additionally, 26% participants reported taking a shower in the morning and evening or engaged in water sport(s) (surfing/swimming) whilst wearing contact lenses at the baseline visit and 27% reported showring in lenses during the clinical trial, and six participants used swimming goggles during water sports activity (**Table 6.6)**. On average 217 ± 112 mL of disinfecting solution was used each month during the two months of the study course.

6.4.5 Clinical trial induced adverse events:

There was no case of solution induced corneal staining with Synergi® disinfecting system during the study. Two adverse events were reported during the study, but both were related to systemic conditions and neither was classified as study related serious ocular adverse event. There was one external hordeolum during the study, but this was deemed to be unrelated either to the use of Synergi® disinfecting solution or to the use of silver lens cases, based on the history of the event obtained by questioning the participant.

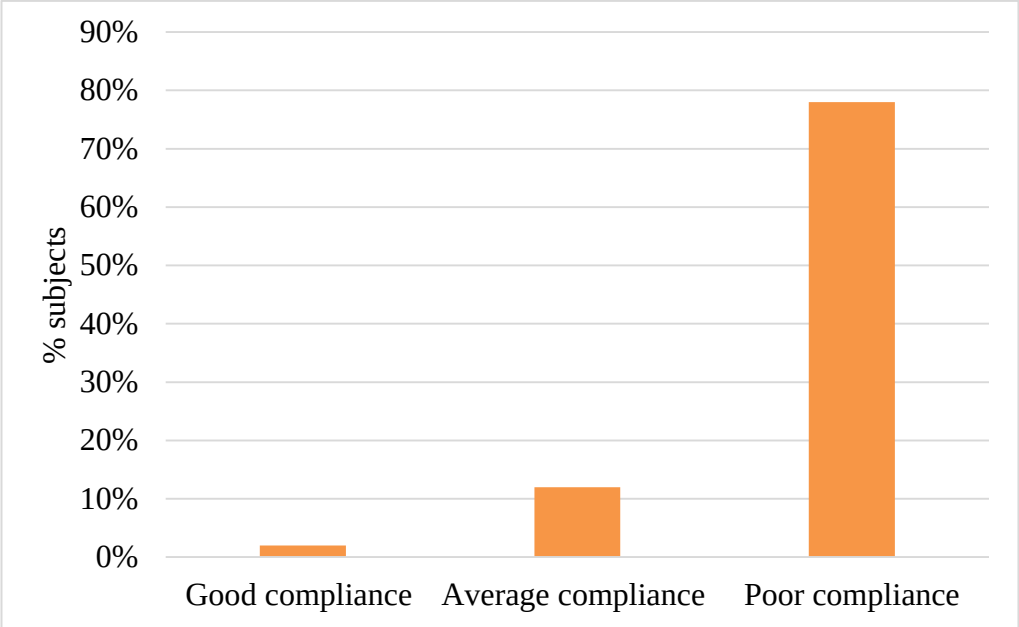


Figure 6-4: The rate of compliance behaviour related to contact lens wear.

Table 6.6: Aspects of non-compliance: summary of comparison of non-compliance frequencies.

Contact lens and lens case procedure	Frequency n (%)		Frequency of non-compliance (%) in previous studies
	Yes	No	
Hand Hygiene			
Hand washing prior to lens handling	94/98 (95%)	8/98 (8%)	11% (Wu *et al*., 2010), 14% (Sokol *et al*., 1990)
Hand washing with soap	83/98 (85%)	11/98 (11%)	35% (Yung *et al*., 2007)
Contact lens hygiene (after lens removal)			
Rubbing lenses	75/98 (77%)	17/98 (17%)	37% (Wu *et al*., 2010)
Rinsing lenses	76/98 (77%)	16/98 (16%)	44% (Dantam *et al*., 2012), 66% *(*Wu *et al*., 2010)
Rubbing and rinsing lenses	68/98 (69%)	10/98 (11%)	69% (Dantam *et al*., 2012)
Contact lens disinfection with-			
Disinfecting solution	89/98 (91%)	5/98 (5%)	52% (Wu *et al*., 2010), 18% (Dantam *et al*., 2012), 77% (Wu *et al*., 2010)
Contact lens case hygiene			
Rinsing of case with Synergi®	85/98 (87%)	9/98 (8%)	41% (Dantam *et al*., 2012)
Rinsing of lens case	96/98 (97%)	2/98 (2%)	74% (Wu *et al*., 2010)
Rinsing lens cases with-			
Disinfecting solution	91/98 (93%)	-	44% (Wu *et al*., 2010)
Water	1/98 (1%)	-	52% (Wu *et al*., 2010)
Rubbing of lens case	35/98 (37%)	55/98 (56%)	70% (Wu *et al*., 2010)
Re-use of solution	32/98 (34%)	60/98 (61%)	13% (Wu *et al*., 2010), 18% (Collins *et al*., 1986)
Air-drying of lens case	68/98 (69%)	23/98 (23%)	23% (Wu *et al*., 2010)
Storage of lens case-			
Bathroom	*57/98 (58%)*	-	-
Bedroom	*33/98 (36%)*	-	-
Kitchen	*1/98 (1%)*	-	-
Water activities (with contact lens)			
Showering	28/98 (29%)	63/98 (64%)	-
Participating in water sports	27/98 (28%)	65/98 (66%)	60% (Bowden & Harknett, 2005; Wu, *et al*., 2010), 56% (Sokol *et al*., 1990)
Using goggles during water sports	6/27 (22%)	-	59% (Wu *et al*., 2010)

a, Italics trend of significant contamination of lens cases based on the storing location of lens storage cases ($p < 0.02$).

6.4.6 Contact lens case contamination:

A total of 48 silver and control cases were included for data analysis. Two of the participants did not return their lens cases at the final visit and one of the participant reported not wearing contact lens for 4 days prior to the final study visit (due to having an external hordeolum), therefore, the last month's data for these participants were excluded from the data analysis due to the protocol violation.

There was a significant difference in the number of lens cases that were contaminated; 13 (27%) silver cases and 17 (35%) control cases ($p < 0.005$; **Figure 6-5**). For silver lens cases, there was a significant difference between the number contaminated with Gram positive bacteria (13%) compared with Gram negative bacteria (2%; $p < 0.05$; **Figure 6-5**). Gram positive and Gram negative bacteria were not isolated from the same lens cases. However, more than one bacterial strains were cultured from 4 (8%) silver lens cases and four, the maximum was cultured from one (2%) contaminated lens case. Similarly 15% of the control lens cases were contaminated with more than one bacterial strain of Gram positive bacteria, and this was significantly higher compared to silver lens cases ($p < 0.005$, **Table 6.7**). Also, 15% of silver and 17% of non-silver cases were contaminated with fungi or yeast (**Figure 6-5)**. Most fungi were recovered in isolation; in only three lens cases were fungi recovered with bacteria. Overall, there were 0.16 ± 0.5 Log_{10} CFU/case (colony forming unit) of microbes in silver and 0.25 ± 0.5 Log_{10} CFU/case in control lens cases ($p > 0.05$; **Figure 6-6**). The range of microbial contamination was 0 to 4.2 ± 1.24 CFU/case for silver cases and 0 to 4.98 ± 1.09 CFU/case for non-silver lens cases, but not significantly different ($p > 0.05$).

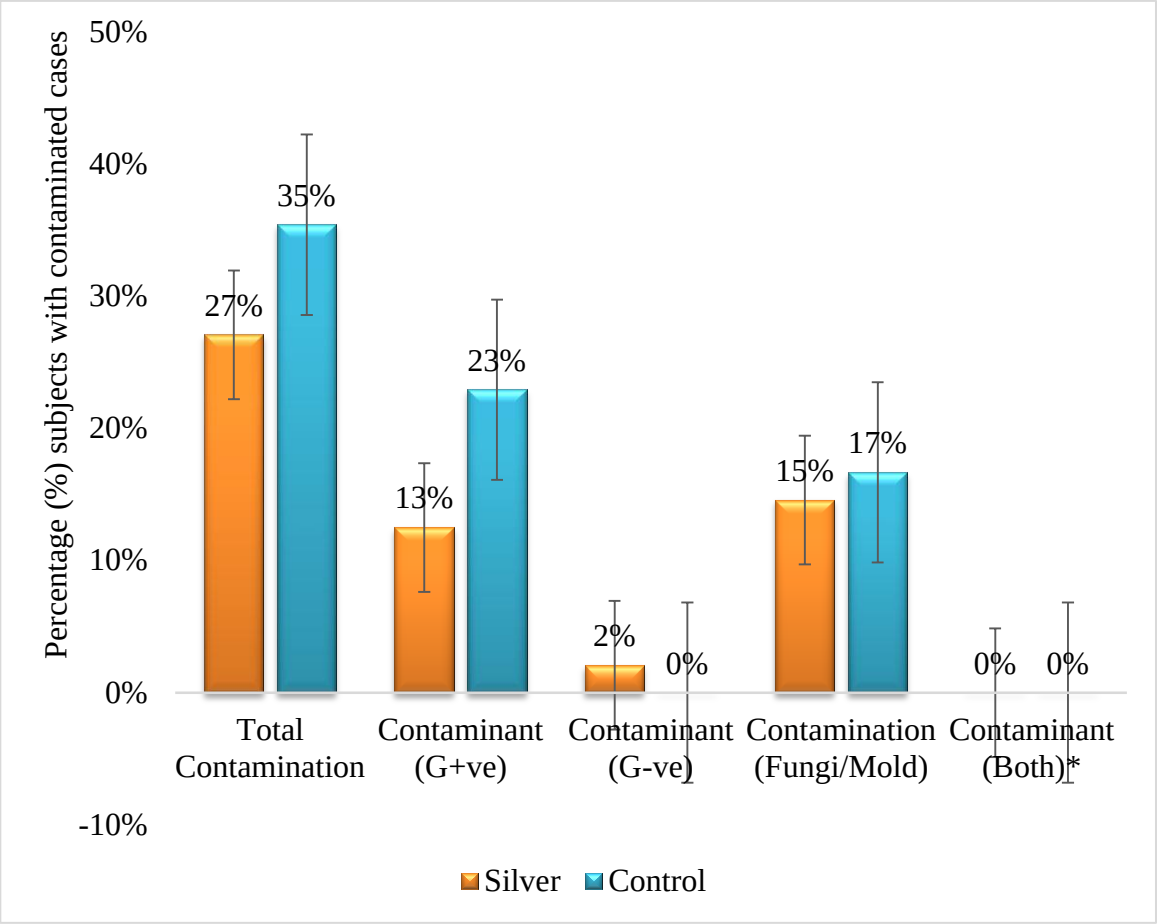


Figure 6-5: Contamination of silver and control lens cases**.**

*, The isolation of Gram positive and Gram negative bacteria together from lens cases represents as "contaminant (both)".

Table 6.7: Types of bacteria and their frequency of isolation from contact lens cases.

	No of organisms	Gram positive	Gram Negative	Fungi
Silver	1 microbial type	2 (4%)	0	7 (15%)
	2 microbial types	2 (4%)	1 (2%)	0
	> 2 microbial types	1 (2%)	0	0
Control	1 microbial type	4 (8%)	0	7 (15%)
	2 microbial types	7 (15%)	0	1 (2%)
	> 2 microbial types	0	0	0

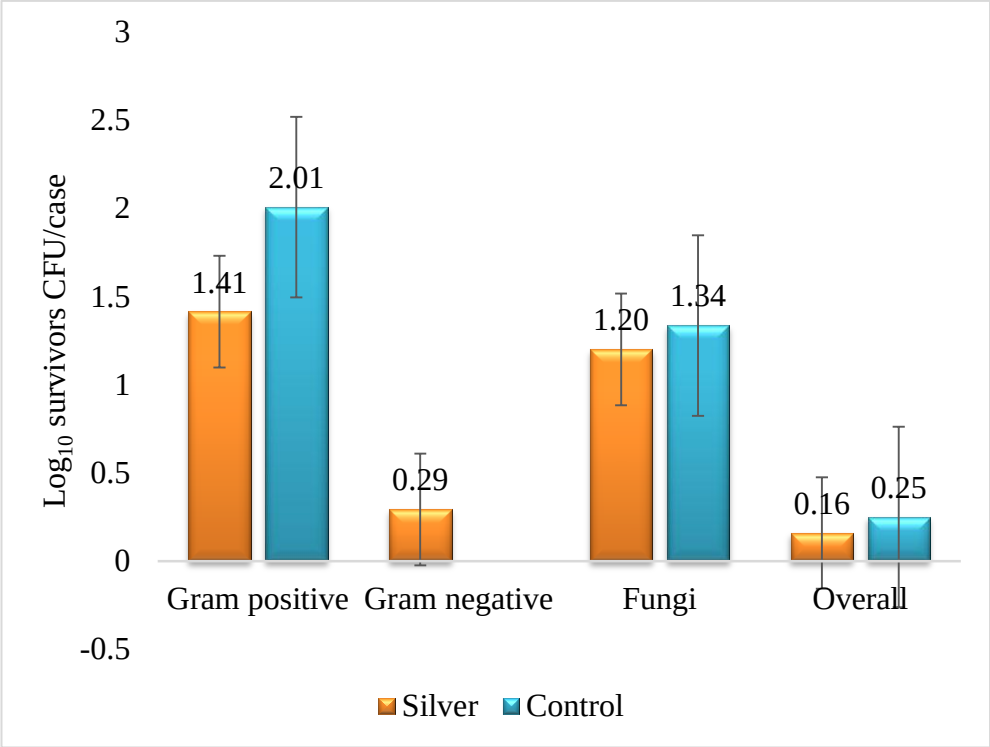


Figure 6-6: Microbial recovery from silver and non-silver lens cases.

Identification of bacterial isolates:

There was no difference in the microbial types isolated from silver or non-silver lens cases (**Table 6.8)**. Coagulase-negative staphylococci were the most commonly isolated Gram positive bacteria from silver barrel cases, with the majority being *Staphylococcus* spp. (15%) followed by *Micrococcus* spp. (4%) (**Table 6.8)**. Overall, the silver-impregnated barrel cases showed low recovery of both Gram positive bacilli (16%), and Gram negative bacteria (2%) (**Table 6.8**).

Table 6.8: Frequency of microorganism recovered from silver and control lens cases.

Group	Organism	n (%)	
		Silver	Control
Gram positive cocci	*Staphylococcus* spp.	7 (15%)	5 (10%)
	Staphylococcus aureus	0	1 (2%)*
	Micrococcus spp.	2 (4%)	3 (6%)
	Planococcus spp.	1 (2%)	0
	Stomatococcus spp.	0	1 (2%)
	Streptococcus spp.	1 (2%)	0
Gram positive bacilli	*Bacillus* spp.	1 (2%)	1 (2%)
	Propionibacterium spp.	1(2%)	0
Gram negative bacteria	*Empedobacter brevis*	1 (2%)	-
	Pasteurella spp.	1 (2%)	-
Fungus	Fungi	5 (10%)	5 (10%)
	Yeast	2 (4%)	3 (6%)

*, considered as pathogenic bacteria isolated form contact lens cases (Mayo *et al*., 1987; McLaughlin-Borlace *et al*., 1998).

Comparison of lens case contamination with lens type, symptomatology, hygiene and compliance:

The use of hydrogel contact lenses was associated with significantly higher levels of case contamination with silver lens cases compared to control cases (**Figure 6-7)**, The hydrogel etafilcon A lens was associated with significantly higher levels of case contamination compared to comfilcon A and senofilcon A silicone hydrogel lenses ($p < 0.05$; **Figure 6-8**). There was no association between case contamination level and ocular discomfort. There was no association between the level of lens case contamination with air-drying of the lens cases after rinsing and the storage location of lens cases ($p = 0.08$; **Figure 6-9)**. The level of contact lens case contamination (CFU/mL) was significantly higher when stored in the bathroom compared to storing the lens case in the kitchen or in bedroom ($p = 0.002$; **Figure 6-9**).

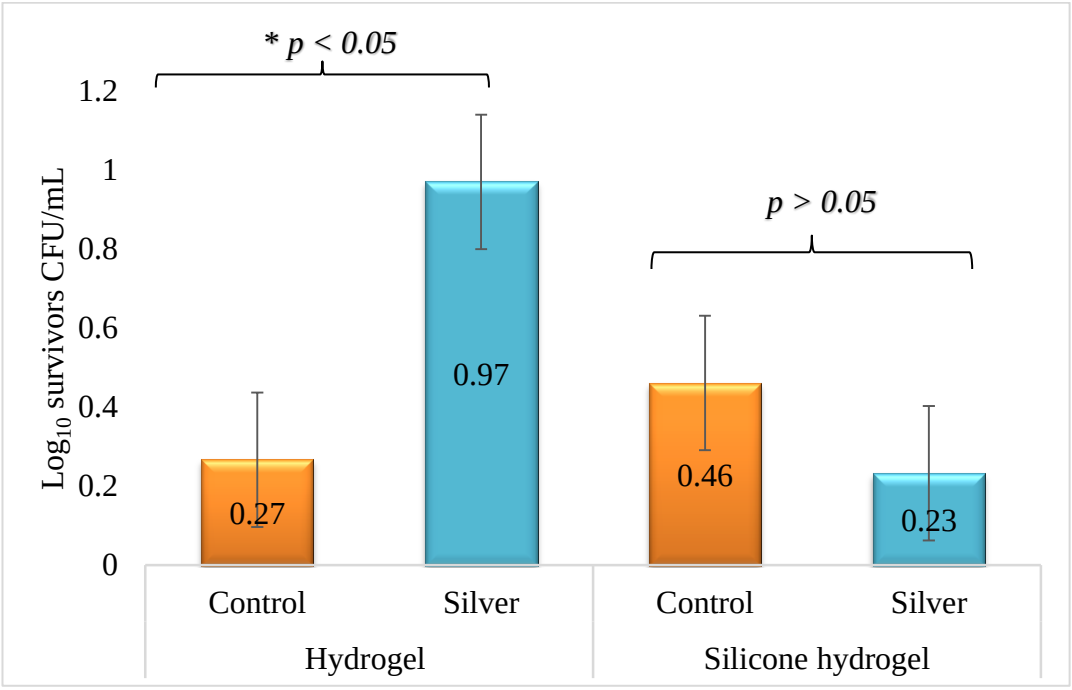


Figure 6-7: Bacteria recovered from silver and control lens cases with silicone hydrogel or hydrogel contact lenses. *, represents $p < 0.05$.

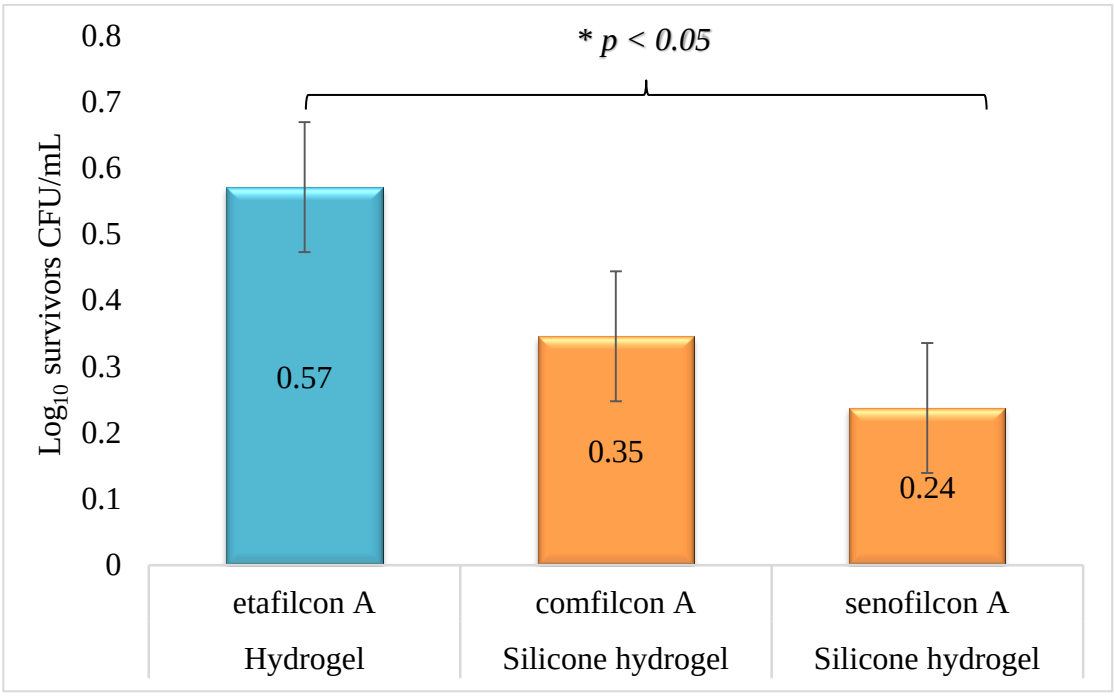


Figure 6-8: Bacteria recovered from lens cases with etafilcon A (hydrogel) and senofilcon A and comfilcon A (silicone hydrogel) contact lenses. *, represents $p < 0.05$.

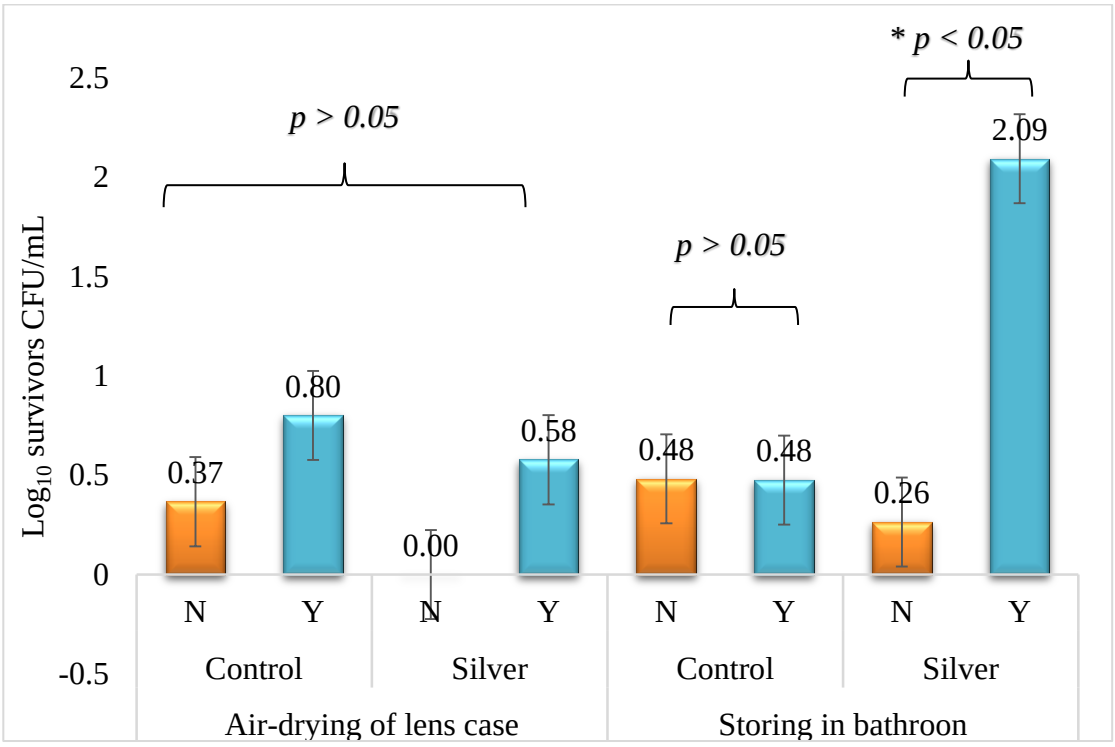


Figure 6-9: Numbers of bacteria recovered from lens cases when air-dried after rinsing and stored in bathroom. *, represents $p < 0.05$.

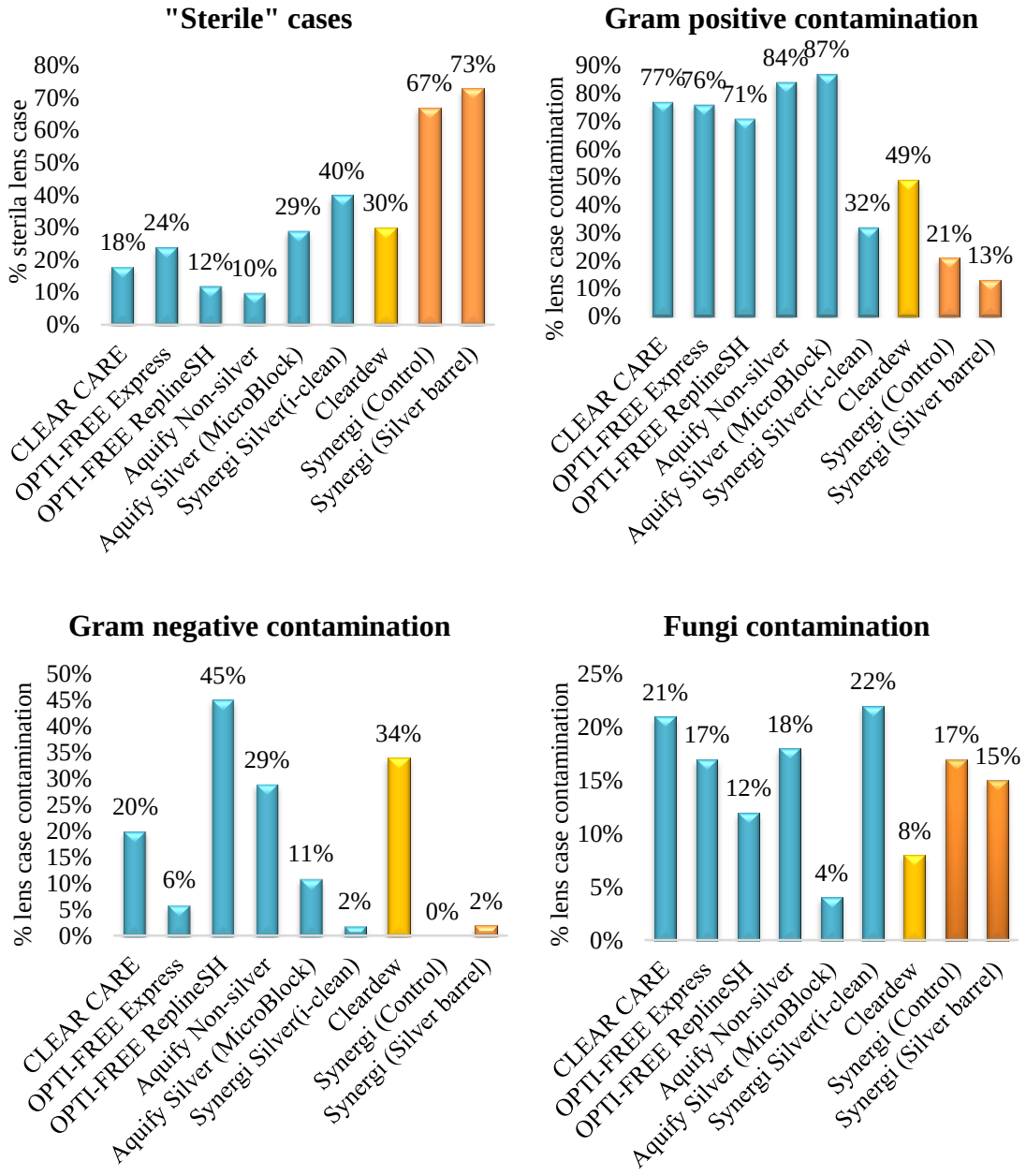


Figure 6-10: Microbial contamination rates of Synergi® and silver lens cases compared with previously published age matched data (Dantam, 2011; Tan *et al*., 2017; Willcox *et al*., 2010).

6.5 DISCUSSION

In the current study the overall rate of microbial contamination of silver barrel lens cases was 27% which is consistent with reported studies with a commercially available (MicroBlock™) silver lens case, where 26% - 38% were contaminated (Amos & George, 2006). However, another study reported 90% contamination with the MicroBlock™ cases and 100% in regular cases (Lakkis and Lakkola, 2006b). Dantam *et al*. reported at least 71% of MicroBlock™ silver-impregnated storage cases were contaminated (Dantam *et al*., 2012). The level of microbial contamination was significantly ($p < 0.005$) lower in silver-impregnated barrel cases (0.16 *vs* 1.7 $\log_{10}$ CFU per case) compared to the MicroBlock™ lens cases (Dantam *et al*., 2012). The differences in the rate of lens case contamination may be attributed to the differences in the processing time following sample collection which ranged from 2 hours to a few days compared within an hour in the present study (Amos & George, 2006; Dantam *et al*., 2012; Lakkis & Lakkola, 2006a). The longer gap in the processing time may reduce the overall rate of lens cases contamination (few days *vs* 4 hours; 26% *vs* 90%; $p < 0.005$) (Amos & George, 2006; Lakkis & Lakkola, 2006a).

The microbial contamination of lens cases was associated with the storage location, air-drying of the lens cases and the involvement of water activities during contact lens wear, which is similar to the previous report (Wu *et al*., 2010). The types of organisms isolated from lens cases also may vary with compliance habits (Wu *et al*., 2010), however no association was found in the current study possibly due to the poor compliance rate among the study participants.

The frequency of Gram positive bacteria, particularly *S. epidermidis* contamination in silver lens cases was significantly reduced compared to control cases which similar to

previous findings (Amos & George, 2006; Lakkis & Lakkola, 2006a). On one occasion, *S. aureus* was recovered from a non-silver lens cases which may be considered as pathogenic organisms in contact lens corneal ulcers and microbial keratitis (Jalbert *et al*., 2000; McLaughlin-Borlace *et al*., 1998; Stapleton *et al*., 2007). There was no recovery of *P. aeruginosa* or *S. marcescens* (Amos & George, 2006; Lakkis & Lakkola, 2006a), which are often associated with contact lens induced microbial keratitis (Cheng *et al*., 1999; Houang *et al*., 2001; Kanpolat *et al*., 1992; Lam *et al*., 2002; Stapleton *et al*., 2007).

Overall, the frequency of lens case contamination in combination with Synergi® disinfecting solution with or without silver cases was reduced ($p = 0.005$; **Figure 6-10**) compared to cleadew™ (Tan *et al*., 2017). Also, the combination of silver cases along with Synergi® disinfecting solution reduced the frequency of multispecies recovery compared to cleadew™ (8% *vs* 30%) but did not eliminate the occurrence of multispecies isolation.

Approximately 15% of silver-impregnated barrel cases were contaminated by fungi which is comparable to an earlier report where 22% of the silver flat cases (i-clean) demonstrated fungal contamination (Dantam, 2011). However, the frequency of fungal contamination in the current study was higher compared to MicroBlock™ silver cases where fungal contamination occurred in only 4% of cases (**Figure 6-10**) when used with AQuify® disinfecting solution (Dantam *et al*., 2012). The toxicity of silver against *Fusarium oxysporum* (Slade & Pegg, 1993) has been reported broadly, but silver-impregnated barrel lens cases were not effective against fungi. However, the frequency of fungi contamination with Synergi® was less than AQuify®, or CLEAR CARE® but not less than cleadew™ ($p < 0.005$), OPTI-FREE® Express® and OPTI-

FREE® RepleniSH® ($p > 0.05$; **Figure 6-10**) (Tan *et al*., 2017; Willcox *et al*., 2010). Unlike previous report stating co-contamination with bacteria (Wu *et al.*, 2010), while the present study co-contamination with bacteria occurred in three silver cases. The recovered fungal isolates were not classified in the present study and the samples were not screened for *Acanthamoeba* spp, which may be important to consider in future studies, given the strong association between *Acanthamoeba* keratitis and contact lens wear (Alfonso *et al*., 2006; Butler *et al*., 2005; Khor *et al*., 2015; Radford *et al*., 1995).

No corneal infiltrative events were observed in this study. This is significantly lower compared to the 27% of participants showing infiltrative events with MicroBlock™ silver-impregnated lens cases and 37% with regular cases (Dantam *et al*., 2012). Additionally, silver-impregnated barrel lens cases along with Synergi® disinfecting solution did not result a solution induced corneal staining, which is similar to the previous findings (Dantam *et al*., 2012). However, the present study was not powered to investigate the incidence of contact lens induced corneal infections, could be the scope to explore in future studies.

Interestingly, hydrogel contact lenses were demonstrated higher contamination in silver lens cases compared to silicone hydrogel lenses, particularly with etafilcon A lenses. However, etafilcon A lenses used with silver barrel cases and Synergi® disinfecting solution did not interfere with the antimicrobial efficacy *in vitro*, as mentioned in Chapter 5. Hence, it will value to conduct further investigations comparing the kinetics of biocide uptake by different contact lens materials with different disinfecting solutions with or without silver cases.

Overall, there was no association between contact lens induced ocular signs/symptoms and the level of microbial contamination in silver barrel cases, despite a previous relationship between the microbial bioburden of contact lens cases and ocular discomfort, dryness, redness and itchiness (Midelfart *et al*., 1996). However, there was no correlation between the level of microbial contamination of lens cases and the contact lens induced ocular symptoms due to the reduced load of microbes in the present study. Participants tended to report reduced end day comfort and increased burning and stinging sensation immediately after lens insertion with the use of Synergi® disinfecting solution with or without silver lens cases. The increased burning and stinging sensation after lens insertion could be due to the low concentration of hydrogen peroxide in Synergi®.

In summary, this chapter has shown silver-impregnated barrel cases reduced overall rate and level of bacterial contamination of Gram positive bacteria. However, further investigations are required to establish whether the use of silver-impregnated barrel cases might limit the contact lens induced adverse responses associated with microbial contamination and to determine whether such benefits are maintained in community studies with large sample size. Also, the mechanism of action of Oxipol™ based disinfection system has not well understood, further investigation is required to measure the effective disinfection time of this disinfection solution which may guide contact lens wearers in maintaining safe and successful lens wear.

Chapter 7:
Summary and Future Work

Chapter 7 Summary and Future Work

7.1 THESIS SUMMARY

Overall, 30% to 85% lens storage cases are contaminated with microbes despite the use of lens care systems (Szczotka-Flynn *et al*., 2010; Wu *et al*., 2015). Lens case contamination has been linked to the development of contact lens induced microbial keratitis (Bates *et al*., 1989; Mayo *et al*., 1987; McLaughlin-Borlace *et al*., 1998). Different strategies have been adopted to reduce microbial colonisation such as the development of easily cleanable lens cases designs, modification in contact lens cleaning and disinfecting solutions and the introduction of antimicrobial lens cases (Dutta & Willcox, 2014; Wu *et al*., 2015). This thesis set out to understand how bacteria colonise cases, the kinetics and patterns of bacterial interactions during colonisation and to determine how antimicrobial strategies might limit storage case contamination, both in laboratory studies and clinical trials.

The initial investigations estimated the rate of lens case in a clinical trial with a relatively newly developed povidone-iodine based disinfecting system (cleadew™) and identified the types of bacterial species that colonized lens cases.

The variation within the microbial species recovered from lens cases, and the isolation of multiple bacterial types from single lens cases, lead to an exploration of bacterial colonisation of lens cases. Information on the mechanism of microbial colonization and biofilm formation in contact lens cases was scarce. Therefore, a major hypothesis in this thesis was to investigate the interactions between bacteria isolated most commonly from lens cases (Chapter 3 and 4).

Additionally, antimicrobial silver-impregnated barrel cases were evaluated for their ability to control microbial colonisation in lens cases. This included laboratory studies of clinical isolates and the standard microbial strains as recommended for the testing of contact lens care products in ISO 14729 guidelines (Chapter 5). Finally, the *in vivo* performance of the silver-impregnated barrel cases was assessed in a human trial to examine the rate and level of microbial colonization and the types of microbes that can be cultured from lens cases during use (Chapter 6). Limiting lens cases contamination may reduce contact lens induced corneal infections. This chapter (Chapter 7) summarises and discusses the key findings of the present work as well as the limitations of the research and future work.

7.2 SUMMARY OF SIGNIFICANT FINDINGS

Use of cleadew™ (povidone-iodine based) multipurpose disinfecting solution resulted in a relatively low level of microbial contamination of cases, with 30% of cases having no culturable microbes (**Table 7.1**) (Willcox *et al*., 2010). Comparison with previously published data (Willcox *et al*., 2010) showed that cleadew™ reduced the frequency of Gram-positive (49%) and fungal (8%) contamination but higher levels of Gram-negative contamination compared to some other disinfecting solutions (**Table 7.1**). There were also examples of cases colonised by multiple types of microbes, with up to 30% of cases having multiple species of microbes isolated from them. This latter finding resulted in a major hypothesis in this thesis which was that the interactions between bacteria may facilitate colonisation of lens cases.

There was very little information in the literature on how microbes colonise contact lens cases, and especially how multispecies colonisation occurs. Experiments were conducted to examine whether strains isolated from the same contact lens case could

coaggregate, cohere or affect the growth of each other. The study found, for the first time, that the following pairs of bacteria could coaggregate, *S. aureus/P. aeruginosa, S. epidermidis/M. luteus,* and *S. epidermidis/A. radioresistens* (**Table 7.2**). Also, those strains of these pairs that had been isolated from the same cases were more likely to coaggregate than strains isolated from different contact lens cases. Furthermore, several of these coaggregating pairs, but not all, could also cohere on contact lens case surfaces (**Table 7.2**). Coaggregation could be involved in cohesion but was not necessary for cohesion to occur. Also, several of these microbial pairs affected the growth of each other. These results suggest many ways which microbes can use to colonise contact lens cases and form multispecies biofilms.

Table 7.1: Rate of lens case contamination and the commonly isolated bacteria recovered from contact lens cases during the clinical trial.

		Key findings			
		cleadew™ disinfecting system	**Control case with Synergi®**	**Silver case with Synergi®**	**P value***
Sample size (n)		41	51	51	
Overall lens case contamination		70% (1.31 ± 1.2)	35% (0.25 ± 0.5)	27% (0.16 ± 0.5)	*0.006*
Bacteria	Gram positive	49% (1.42 ± 0.6)	17% (1.66 ± 0.7)	13% (1.30 ± 0.7)	*0.005*
	Gram negative	34% (2.50 ± 2.1)	0% -	2% (2.04 ± 0.02)	*0.005*
	Combination†	69% (2.76 ± 1.8)	0% -	0% -	*0.001*
	Multispecies†	30% (2.25 ± 2.5)	15% (1.31 ± 0.5)	8% (1.08 ± 0.5)	*0.006*
Fungi		8% (1.42 ± 0.5)	17% (0.22 ± 0.6)	15% (0.19 ± 0.5)	*0.005*
		Commonly isolated organisms			
Gram positive bacteria					
Coagulase negative staphylococci [a]		52% (3.49 ± 1.5)	10% (1.41 ± 0.7)	15% (1.02 ± 0.5)	*0.006*
Micrococcus spp.[a]		15% (3.21 ± 1.7)	6% (2.38 ± 1.5)	4% (1.66 ± 1.2)	*0.005*
Enterococcus spp.		11% (1.75 ± 1.1)	0% -	0% -	*0.005*
Gram negative bacteria					
Serratia marcescens		12% (5.54 ± 1.8)	0% -	0% -	*0.005*
Acinetobacter spp.[a]		10% (2.54 ± 1.2)	0% -	0% -	*0.005*
Enterobacter spp.		10% (1.60 ± 0.7)	0% -	0% -	*0.005*

a, these organisms were isolated frequently together from the lens cases of asymptomatic wearers.

†, combination and multispecies of bacteria represent the isolation of Gram positive and Gram negative bacteria together and multispecies of bacteria represents the isolation of more than one bacterial colony from same lens case, respectively.

*, significant difference was seen in the rate and level of lens case contamination between cleadew™ and silver lens cases with Synergi® disinfecting solution.

Table 7.2: Bacterial interactions among contact lens case contaminants.

Species of bacteria	**Bacterial interaction**		
	Coaggregation (%)	**Cohesion**	**Growth**
***S. aureus* 31* + *P. aeruginosa* 6294**	62 ± 3	No	Inhibited
***S. aureus* 31* + *P. aeruginosa* Paer1**	58 ± 2	No	Inhibited
S. epidermidis* 22-1* + *A. radioresistens* 22-1	54 ± 5	Yes	Enhanced
***S. epidermidis* 22-1 + *M. luteus* 22-1**	50 ± 3	Yes	No effect

*, the bacteria that was affected.

Subsequent to these findings the effects of a different disinfectant in a multipurpose solution and silver in lenses cases were examined to determine if these affected microbial colonisation and production of multispecies biofilms.

The use of Synergi® disinfecting solution, which contains an oxidative disinfectant, in combination with silver lenses cases resulted in a significant reduction in lens case contamination. Approximately, 73% of lens cases had no culturable microbes (**Table 7.1**). Furthermore, the combination of silver barrel cases with Synergi® disinfecting solution significantly ($p < 0.005$) reduced the chance of multispecies isolation from the same lens cases (8% *vs* 15%; **Table 7.1**). Overall, the bactericidal efficacy of Synergi® disinfecting solution was better compared to cleadew™ *in vivo* with the exception of fungal contamination. The use of silver-impregnated barrel lens cases

significantly reduced the rate and level of microbial contamination in lens cases compared to flat silver cases (MicroBlock™ and i-clean) (Dantam *et al*., 2012).

These changes to contact lens case contamination rates with the iodine or oxidative disinfectants also resulted in reductions in contact lens induced corneal inflammation. The iodine solution resulted in a low corneal infiltrative event rate of 0.8% per 100 participant-months. The use of Synergi® disinfecting solution resulted in no corneal infiltrative events. Surprisingly, given the data of the current study, Synergi® contact lens solution has been phased out by the manufacturer starting from December 2017 (https://www.opticianonline.net/news/coopervision-discontinues-contact-lens-solution). According to the manufacturer, they took this decision due to a forthcoming research paper which affect the reputation and sales of Synergi® disinfecting solution. No further explanation was given, but it will be interesting to see whether this decision is based on microbial problems with the solution. In the present study, the efficacy of Synergi® disinfecting solution with or without silver lens case was found relatively low against fungi. Perhaps, an issue with fungi contamination could be the reason for the termination of the product.

In summary, it appears that certain bacteria commonly found in storage cases can cohere, that is the presence of one bacterial type increases the ability of another to attach and this can be facilitated by coaggregation, the direct adhesion of bacterial cells to each other. The study findings may help to identify better approaches to prevent biofilms by targeting specific bacteria which accelerate the process of biofilm formation. Also, the use of coaggregation inhibitory substances may be an approach to inhibit bacterial coaggregation to reduce the level of contact lens case contamination. Although, this requires further investigations both in laboratory studies and in clinical

trials. The outcome of this part of the thesis may lead to new approaches to eliminate contact lens case contamination, and consequently minimize contact lens associated ocular complications.

7.3 IMPLICATION OF THE RESEARCH

Demonstrating that coaggregation, cohesion and growth of bacteria can affect colonisation of contact lens cases has the potential to be used to develop new excipients in contact lens disinfecting solutions that interfere with these processes. Thus, it may result in further reductions in the rate of colonisation of lens cases. For example, substances that inhibited coaggregation or isolation and addition of growth interfering substances might prove useful excipients.

Another practical implication would be to include scaffold/primary coloniser organisms from the lens cases in the list bacteria that should be tested under ISO guidelines for evaluating the antimicrobial efficacy of the contact lens disinfecting solutions or antimicrobial lens cases. Furthermore, it would be interesting to assess the antimicrobial efficacy of disinfecting solutions using combinations of bacterial pairs instead of using a single type of bacteria.

Furthermore, the effectiveness of the combination of an oxidative disinfectant with silver in lens cases argues for the continued development of these systems. The combination of silver cases along with the case matched contact lens cleaning and disinfecting solution might reduce the multispecies isolation from lens cases.

7.4 LIMITATIONS OF THE STUDY AND FUTURE WORK

The scope of this research was to estimate the rate of lens cases contamination during daily lens wear. During the clinical trials, the habitual lens case and disinfecting solution were not collected from participants at the baseline visit. This may be a limitation as participants, whilst advised not to use these old cases or solutions, may have continued to use them occasionally. New studies should collect and dispose of these old cases and solutions to ensure study compliance.

Investigating the antimicrobial efficacy of silver-impregnated barrel lens cases kept dry or wet between use should be the part of future research. Silver-impregnated lens cases reportedly perform better when kept wet (Dantam *et al*., 2012).

The compatibility of silver-impregnated barrel lens cases with different types of disinfecting solutions apart from Synergi® disinfecting solution was not investigated. Whilst manufacturers recommend using particular lens cases with particular MPDS, it would be interesting to determine whether the silver lens cases are effective with other MPDS, as this might encourage development of new silver lenses cases by more manufacturers, and ultimately lead to a reduction in keratitis associated with lens wear.

The present study did not analyze lens cases for the presence of *Acanthamoeba*, and new studies should include this analysis – even though cases are rarely contaminated with this microbe (Willcox *et al*., 2010). Also, the recent withdrawal of the product from the commercial market has raised the question that whether this decision is based on microbial problems particularly against *Acanthamoeba* with the solution. The microbial analysis of the clinical trials relied on culture of microbes followed by identification of the genus/species of bacteria, using the microbial metabolism and

biochemical properties for silver lens case clinical trial and 16s RNA technique for cleadew™ disinfecting system trial. However, it is clear from many studies (Dong *et al*., 2011; Wiley *et al*., 2012) using non-culture techniques that culture underestimates the types of microbes that are present. Therefore, future studies should use DNA techniques such as 16s RNA analysis to analyze the microbiome of lens cases, and the effect on the microbiome of disinfectants and antimicrobials such as silver.

This study explored microbial consortia in contact lens cases which may be useful in determining the natural history and susceptibility to removal of biofilm from lens cases. High resolution imaging to visualise the 3D structures and the natural history of biofilm would complement the viable microbial recovery. Fluorescent *in situ* hybridization (FISH) can be performed with rRNA-targeted oligonucleotide probes and micro-autoradiography (Møller *et al*., 1998). In mixed-culture biofilms FISH can be used to identify gene expression. The use of Green Fluorescent Protein tagged proteins of microbial isolates could be used to investigate structure-function relationships in microbial communities (Møller *et al*., 1998). A better understanding of these interactions, both at molecular and biophysical levels, could lead to novel intervention strategies for controlling pathogenic biofilm formation in lens cases.

The sample size of the present clinical trial was not powered to estimate the incidence rate of contact lens induced corneal adverse events. Considering previous reports of the association between contact lens case contamination and the incidence of contact lens induced microbial keratitis (Bates *et al*., 1989; McLaughlin-Borlace *et al*., 1998; Stapleton *et al*., 1995), new research should probably also examine the rate of contact lens induced corneal adverse responses.

7.5 CONCLUSION

The thesis has contributed to the understanding of the mechanisms of interactions between bacterial species in contact lens cases. A complex series of events occur (adhesion, coaggregation, cohesion and growth) which may be involved in multispecies biofilm formation in contact lens cases. Understanding these may help produce new technologies to control lens case contamination.

Silver-impregnated barrel lens cases demonstrated good antimicrobial efficacy against a broad range of bacterial species including multidrug-resistant *P. aeruginosa* and *S. aureus* strains and the standard strains as per the ISO guidelines. Finally, during the human clinical trial the use of silver-impregnated barrel cases resulted in a reduction in the overall rate of lens case contamination particularly in the reduction of the recovery of Gram positive bacteria. Thus, the use of silver-impregnated barrel lens cases with an appropriate lens care system can be an alternative to reduce the lens case contamination in use and further to prevent microbial complications of daily wear contact lenses.

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Appendix A

Publications and Presentations

PUBLICATIONS

Accepted:

- Bacterial coaggregation among the most commonly isolated bacteria from contact lens cases. Datta A, Stapleton F, Willcox MDP. *Invest Ophthalmol Vis Sci*. 2017; 58(1):50-58.
- Clinical outcomes and contact lens case contamination using a povidone iodine disinfection system. Tan J, Datta A, Wong K, Willcox MDP, Vijay AK. *Eye & Contact Lens*. 2017 Mar 21. doi: 10.1097/ICL.0000000000000385.
- Bacterial coaggregation and cohesion among the isolates from contact lens cases. Datta A, Stapleton F, Willcox MDP. (under review at *Invest Ophthalmol Vis Sci*)

PRESENTATIONS

Oral

- ***Datta A***, Stapleton F, Willcox MDP. (2017) "Understanding bacterial behaviour that may influence biofilm formation in contact lens cases". International Society of Contact Lens Research (ISCLR), *Oregon, United States of America.*

Poster

- ***Datta A***, Stapleton F, Willcox MDP (2018) "Bacterial coaggregation and cohesion among the isolates from contact lens cases". Association for Research in Vision and Ophthalmology (ARVO). *Hawaii, United States of America*.
- ***Datta A***, Stapleton F, Willcox MDP. (2017) "Understanding bacterial behaviour that may influence biofilm formation in contact lens cases". International Society of Contact Lens Research (ISCLR), *Oregon, United States of America*.

- ***Datta A***. Willcox MDP, Stapleton F. (2017) "Antimicrobial efficacy of silver copolymerized barrel lens cases". Association for Research in Vision and Ophthalmology (ARVO). *Baltimore, United States of America*.

- ***Datta A***. Willcox MDP, Stapleton F. (2017) "Antimicrobial efficacy of silver copolymerized barrel lens cases along with disinfecting solution". International Cornea and Contact Lens Congress (ICCLC). *Sydney, Australia*.

- ***Datta A***, Stapleton F, Willcox MDP. (2016) "Coaggregation of bacteria isolated from contact lens cases". American Academy of Optometry (AAO). *Anaheim, United States of America.*

SCHOLARSHIPS AND GRANTS

Scholarships

- Tuition Fee Remission Scholarship (TFRS), University of New South Wales, 2014 - 2017
- Post graduate student research scholarship, University of New South Wales, 2014 - 2017
- Postgraduate Research Student Support (PRSS) scholarship, 2016

Grants

- **International Society for Contact Lens Research (ISCLR)**

  ISCLR International student travel grant, 2017
- **Association for Research in Vision and Ophthalmology (ARVO)**

  ARVO International student travel grant, 2017
- **American Academy of Optometry (AAO)**

  AAO Student Travel grant, 2016

Appendix B (1)

Participant Informed Statement and Informed Consent Form

CLEADEW™ SOLUTION CLINICAL TRIAL HREC APPROVAL NO. (HREC 14271)

School of Optometry and Vision Science

HREC Approval No: HC14270

UNSW, SYDNEY

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM

A 3 month dispensing study of First Care cleaning and disinfecting solution in daily wear, frequent replacement soft contact lens wearers

Principal Investigator: Dr Jacqueline Tan

Introduction

You are invited to take part in this research project, which is called "A 3 month dispensing study of First Care cleaning and disinfecting solution in daily wear, frequent replacement soft contact lens wearers". You have been invited because you currently wear frequent replacement soft contact lenses on a regular basis. Your contact details were obtained either from the School of Optometry and Vision Science database because you nominated to be on our research database, or via your response to a study advertisement.

This Participant Information Sheet/Consent Form tells you about the research project. It explains the processes involved with taking part. Knowing what is involved will help you decide if you want to take part in the research.

The School of Optometry and Vision Science is being paid by the Co-sponsor (Ophtecs Corporation) to undertake this study at UNSW.

What is the purpose of this research?

Proper contact lens care is vital for protection of the eyes. Without daily cleaning and disinfection, bacteria and other microbes can grow, thereby increasing the risk of eye infections to occur. First Care is the only product on the market of its kind that uses Povidone-iodine as a disinfectant, and has high activity against acanthamoeba, which

is a potentially vision threatening organism in eye infections. Povidone-iodine is the most commonly used medical disinfectant, and First Care has been shown to be a safe and efficient care product for all types of frequent replacement soft contact lenses (including silicone hydrogel lenses). First Care is approved for sale and distribution in Japan, but is not currently approved in Australia. We hope to learn how effective this solution is for reducing microbial contamination (e.g. bacteria and fungi) of contact lens cases, over a 3 month period, and to compare the results to other commercially available soft contact lens cleaning and disinfecting solutions.

Why have I been invited to participate in this research?

You have been invited to participate in this research because you: are at least 18 years of age; currently wear frequent replacement soft contact lenses; are willing to wear your lenses during the day (no overnight wear) for a minimum of 4 days per week (on average) for the duration of the study; are willing to use the First Care cleaning and disinfecting solution for the duration of the study; have no known sensitivity/allergy to iodine; have no known reasons why you cannot safely wear contact lenses have not undergone eye surgery within the previous 12 weeks; are not currently enrolled in any other clinical trial and; are not pregnant (if female).

Description of study procedures and risks

If you decide to participate, we will ask that you continue to wear your regular soft contact lenses on a daily wear basis (no overnight wear), at least 4 days per week (on average) and replace them at the usual frequency prescribed by your eye care practitioner. However, we ask that you start wearing a new (fresh) pair of lenses beginning on Day 1 of this study. You will be provided with First Care, which is to be used to replace your regular lens care solution and lens case, for cleaning, disinfecting and storing your contact lenses after daily lens removal. There are a total of 3 scheduled study visits over a period of approximately 3 months: Day 1, 1 Month and 3 Months. Visits will take approximately 1 hour to complete.

Before any study-related procedures are performed, you will be asked to read and sign this Participant Information and Informed Consent Form if you wish to participate. The table below gives details of the procedures that will take place before and during the study. All study procedures are standard tests used in routine Optometric practice, with the exception of used contact lens cases being collected for analysis of microbial contamination.

Procedures/ Data (Y/N)	Visit 1 BL	Visit 2 (1M from BL)	Visit 3/Study Exit (3M from BL)	Unscheduled / Adverse Events
Visit Window	N/A	± 7 days	± 14 days	N/A
Informed Consent	Y	N	N	N
Meet Inclusion/Exclusion Criteria	Y	N	N	N
Ocular and Medical History, Medications, Demographics	Y (I)	N	N	N
Updated History, Symptoms and Problems	N	Y (I)	Y (I)	Y (I)
Vision Tests (Visual Acuity)	Y (M)	Y (M)	Y (M)	Y (M)
Slit-Lamp Biomicroscopy: Lens fit, Anterior ocular health including evaluation of cornea & conjunctiva with fluorescein	Y (M)	Y (M)	Y (M)	Y (M)
Questionnaires and Rating scales	Y (M)	Y (M)	Y (M)	*
Lens Case Returns	N	Y (I)	Y (I)	Y (I)
Return Unused Study Lens Care Product	N	N	Y (I)	*
Adverse Event Data	(Y)**	(Y)**	(Y)**	Y

Y = Yes, required information, N = No, not required, I = Interview, M = Measurement or observation

* *At optometrist's discretion* *M = Month*
** *If adverse event detected at time of visit*

If you or the study optometrist feel that you need to come in at another time between scheduled visits, another appointment will be scheduled for you. Additional testing may be required at the discretion of your optometrist.

Problems associated with the use of your contact lenses such as discomfort, redness of the eye, light sensitivity or blurry vision, remain the same as if you were not participating in this study. However, additional risks associated with use of the study lens care product with your current contact lenses may include:

 Eye irritation (less than 1in 100 people will experience this)

 Eye itching (less than 1 in 100 people will experience this)

 Eye/eyelid swelling (less than 1 in 100 people will experience this)

 Eye redness (less than 1 in 100 people will experience this)

The fluorescein dye may in rare cases, cause eye stinging or burning. In the rare instance that you are allergic to the study lens care product or the fluorescein dye, you may experience redness, itching and watering around the eyes.

Signs of ocular allergy will be treated by immediate discontinuation of (i) use of the study lens care product (ii) participation in the study and (iii) contact lens wear until ocular signs and symptoms have returned to normal. Topical and/or oral antihistamines may be prescribed if deemed appropriate. To monitor for any adverse signs and symptoms, thorough history-taking will be conducted, and detailed evaluation of ocular health will be performed at the scheduled follow-up visits. You will also be provided with a contact number to reach the study investigator, in case an ocular emergency occurs at home.

There are no known risks associated with any of the study products that would require emergency medical care. However, in the unlikely event of a medical emergency, you are advised to contact a medical professional in the first instance. If time allows, you should immediately discontinue use of the study lens care product and remove your contact lenses.

In addition, there are no known risks to women who are pregnant or the unborn foetus. However, hormonal changes are known to affect the eyes, and therefore it is considered appropriate to exclude this population.

What are the possible benefits of taking part?

You may be at a lower risk of developing acanthamoeba related eye infections, which are in any case very rare (estimated annualized incidence rates: approx. 2 cases per million contact lens wearers in US), while using the study lens care product for the duration of the study. However, we cannot and do not guarantee or promise that you will receive any benefits from this study.

What are the alternatives to participation?

There are a number of alternate commercially available contact lens cleaning and disinfecting solutions on the market. As you are an existing frequent replacement soft contact lens wearer, you are probably already using one of these mulitpurpose or hydrogen peroxide-based cleaning systems.

Confidentiality and disclosure of information

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission, except as required by law. If you give us your permission by signing this

document, we plan to publish the results in local and International journals and present the results at local and International conferences and meetings. In any publication, information will be provided in such a way that you cannot be identified.

The Therapeutic Goods Administration requires that your study records must be retained for 15 years after the completion of the study. After this period, the records will be shredded, incinerated or securely recycled.

Recompense to participants

At each visit, you will be reimbursed $20 for your travel and refreshment costs incurred in attending your study visits. There are no costs associated with participating in this research project. You do not have to pay for the study visits, tests or study lens care products that are provided to you as part of your participation in the study.

Complaints

Complaints may be directed to the Ethics Coordinator, The University of New South Wales, SYDNEY 2052 AUSTRALIA (phone (02) 9385 4234, fax (02) 9385 6222, email: humanethics@unsw.edu.au. Any complaint you make will be investigated promptly and you will be informed out the outcome.

Feedback to participants

A short summary of the results will be provided, should you indicate a desire to receive feedback on the study at your final study visit, once the study findings have been published.

Your consent

Your decision whether or not to participate will not prejudice your future relations with the University of New South Wales. If you decide to participate, you are free to withdraw your consent and to discontinue participation at any time without prejudice. If you decide to withdraw from this research project, please notify a member of the research team before you withdraw. A member of the research team will inform you if there are any special requirements linked to withdrawing. The Investigator may also permanently discontinue your participation in the study if in the Investigator's opinion it is in your best interest. Examples include: persistent clinical trial-related symptoms/complaints that are not correctable, or a serious adverse event/serious adverse device event that is eye/lens/solution related. Repeated failure to follow clinical trial requirements and instructions may also result in permanent discontinuation from the study.

In the event of study withdrawal or early exit, you will be asked to attend a final study exit visit, to ensure that your eyes are healthy and to return any unused study lens care product. When withdrawing please complete the attached *Revocation of Consent Form.*

If you have any questions, please feel free to ask us. If you have any additional questions later, Dr Jacqueline Tan (02) 9385 6551, will be happy to answer them.

You will be given a copy of this form to keep.

THE UNIVERSITY OF NEW SOUTH WALES

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM (continued)

A 3 month dispensing study of First Care cleaning and disinfecting solution in daily wear, frequent replacement soft contact lens wearers

Principal Investigator: Dr Jacqueline Tan

Declaration by Participant

□ I have read the Participant Information Sheet, or someone has read it to me in a language that I understand.

□ I understand the purposes, procedures and risks of the research described in the project.

□ I have had an opportunity to ask questions and I am satisfied with the answers I have received.

□ I freely agree to participate in this research project as described and understand that I am free to withdraw at any time during the project without affecting my future care.

□ I understand that I will be given a signed copy of this document to keep.

……
…….

Signature of Research Participant Signature of Witness

……
…….

(Please PRINT name) (Please PRINT name)

……
…….

Date Nature of Witness

REVOCATION OF CONSENT

A 3-month dispensing study of First Care cleaning and disinfecting solution in daily wear, frequent replacement soft contact lens wearers

Principal Investigator: Dr Jacqueline Tan

I hereby wish to **WITHDRAW** my consent to participate in the research proposal described above and understand that such withdrawal **WILL NOT** jeopardise any treatment or my relationship with The University of New South Wales.

……
……..

Signature Date

……

Please PRINT Name

The section for Revocation of Consent should be forwarded to:

Principal Investigator: Dr Jacqueline Tan

School of Optometry and Vision Science

The University of New South Wales

Level 3, Rupert Myers Building, North Wing

Gate 14, Barker Street,

Sydney NSW 2052

E-mail: jacqueline.tan@unsw.edu.au

Appendix B (2)

Participant Informed Statement and Informed Consent Form

SILVER-IMPREGNATED BARREL LENS CASES CLINICAL TRIAL (HREC APPROVAL NO. HC#16961)

The University of New South Wales, School of Optometry and Vision Science

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM
Participant Group: Contact lens wearers
Title of project: Evaluation of silver contact lens storage cases in daily wear soft contact lens wear.
Chief Investigator: Prof Fiona Stapleton
The study is being carried out by the following researchers:

Role	Name	Organisation
Chief Investigator	Prof Fiona Stapleton	School of Optometry and Vision Science, University of New South Wales
Co-Investigator/s	Prof Mark Willcox	School of Optometry and Vision Science, University of New South Wales
Student Investigator/s	Ananya Datta (M. Phil in Optometry and Vision Science)	School of Optometry and Vision Science, University of New South Wales
Research Funder	This research is not funded by an external organization.	

1. What is the research study about?

You are invited to take part in this research study to determine how often contact lens storage cases become contaminated in use and the types of organisms recovered from lens cases. You will be eligible to take part in this study, if you meet the following criteria:

Key inclusion criteria:

• Be at least 18 years of age;

• An existing contact lens wearer (hydrogel or silicone hydrogel) not currently wearing daily disposable contact lenses;

• Willing to wear the contact lenses on a daily wear basis for a minimum of 4 days per week (on average) for the duration of the study;

• Willing to use the study prescribed contact lens case and disinfecting solution for the duration of the study.

Key exclusion criteria:

• Daily disposable or rigid gas permeable lens wearer (including orthokeratology);

• Non-contact lens wearer;

• Self-reported metal/silver sensitivity/allergy;

• Any active corneal infection, ocular disease or systemic disease that would affect wearing of contact lenses;

• Use of or need for any systemic or topical medications which may alter normal ocular findings/are known to affect a participant's ocular health/physiology or contact lens performance either in an adverse manner or risk providing a false positive;

• Eye surgery within 12 weeks immediately prior to enrolment for this trial;

• Contraindications to contact lens wear;

• Currently enrolled in another clinical trial.

The study aims to examine the rate of contamination of silver impregnated lens cases compared with non-silver lens cases and also to evaluate the level of biofilm formation on the surface of lens cases surfaces. If you decide to participate, you will use the silver impregnated barrel shaped lens case and non-silver lens case (each for one month) along with the recommended multipurpose disinfecting solution. Routine assessment of contact lens fitting, and anterior eye health will be conducted at each visit, and lens cases will be collected for microbial analysis at the 1 and 2 month visits. You will be required to complete a questionnaire relating to your contact lens use at 1st and 2nd month visits.

2. Do I have to take part in this research study?

Participation in this research study is voluntary. If you don't wish to take part, you don't have to. Your decision will not affect your relationship with The University of New South Wales or the School of Optometry and Vision Science.

This Participants Information Statement and Consent Form tell you about the research study. The participant's informed consent and the project information form will be emailed to you. It explains the research tasks involved during this study. Knowing what is involved will help you decide if you want to take part in the research.

Please read this information carefully. Ask questions about anything that you don't understand or want to know more about. Before deciding whether or not to take part, you might want to talk about it with a relative or friend.

If you decide you want to take part in the research study, you will be asked to:

- Sign the consent form;
- Keep a copy of this Participant Information Statement;

3. What does participation in this research require, and are there any risks involved?

Participants will be asked to use the recommended contact lens storage cases and multipurpose solution for storing their contact lenses and to a self-administered questionnaire about their contact lens use. Participants will visit the clinic three times for this study. In the first visit they will be supplied one type of lens case and in the 2nd visit the other type along with the disinfecting solution. The routine assessment of lens fit, and anterior eye health will be conducted at each visit as described earlier. Along with that the quantity of left over solution in the bottle will be also measured. This study will be conducted at the School of Optometry and Vision Science, UNSW. At the end of 1st and 2nd month visits they will complete the questionnaire and we expect this activity will take approximately 20-30 mins to complete.

During the trial, it is possible, although rare that participants may have an allergic reaction to the Synergi (Sauflon) multipurpose disinfecting solution or to fluorescein dye that is instilled onto the surface of the eye during routine ocular health assessment. (less than 1in 100)

All potential participants will be questioned regarding any history of allergy to multipurpose solutions, dye or silver prior to enrolment in the study. Any participants at risk of silver sensitivity will be excluded from the study.

Signs of ocular allergy (e.g. redness, itching and watering) will be treated by immediate discontinuation of (i) use of the study lens care product (ii) participation in the study and (iii) contact lens wear until ocular signs and symptoms have returned to normal. Topical and/or oral antihistamines or other treatment may be prescribed by the investigation if deemed appropriate.

A detailed evaluation of ocular health will be performed at the scheduled follow-up visits to monitor for any adverse signs and symptoms. In the event that you experience any problems with any of the study procedures or the study contact lens cases or disinfecting solution, you should notify the study optometrist immediately by phone on (02) 9385 4536. For out of hour's emergency contact please contact your local emergency services, Sydney eye hospital (T: 02 9382 711).

The schedule of visits and the data requirements for each visit including the baseline visit are shown in the table below.

Procedures (Y/N)	*Visit 1* BL	*Visit 2* (1M from BL)	*Visit 3/Study Exit* (2M from BL)	Unscheduled / Adverse events
Visit Window	N/A	± 7 days	± 7 days	**N/A**
Informed consent	Y	N	N	**N**
Meet inclusion/exclusion criteria	Y	N	N	**N**
Ocular and medical history, medications, demographics	Y (I)	N	N	**N**
Updated history, symptoms and problems	N	Y (I)	Y (I)	**Y (I)**
Vision tests (Visual acuity)	Y (M)	Y (M)	Y (M)	**Y (M)**
Slit-lamp biomicroscopy: Lens fitting, anterior ocular health including evaluation of cornea & conjunctiva with fluorescein	Y (M)	Y (M)	Y (M)	**Y (M)**
Questionnaires and Rating scales	N	Y (M)	Y (M)	*****
Lens Case and Solution Bottle Returns	N	Y (I)	Y (I)	**N**
Return Unused Study Lens Care Product	N	N	Y (I)	*****
Adverse Event Data	**(Y)****	**(Y)****	**(Y)****	**Y**

Y = Yes, required information, N = No, not required, I = Interview, M = Measurement or observation

* *At optometrist's discretion, M = Month*

** *If adverse event detected at time of visit*

4. Will I be paid to participate in this project?

You will be provided with a gift of $10 voucher on 2nd and 3rd visits, to compensate for your travel and refreshment costs incurred in attending the study visits.

5. What are the possible benefits to participation?

We hope to use information we get from this research study to benefit others who wear contact lenses. While silver cases are available in Europe, they are not currently available in Australia. Silver is active against both Gram positive and Gram negative bacteria and the slow release of silver ions inhibits bacterial growth by multiple

methods. However, there are limited available data on the clinical performance of silver-impregnated storage cases. This study will help us to estimate how these lens cases perform in use. This study will help to understand the relationship between compliance and contamination among daily wear soft contact lens wearers.

6. What will happen to information about me?

By signing the consent form, you consent to the research team collecting and using information about you for the research study. We will keep your data for 15 years. Any information obtained in connection with this research study that can identify you will remain confidential. If you agree to participate in this study, the responses you provide to the questionnaire will be stored in a secure location at UNSW Australia. All participants will be given a unique study identification code. Data will be de-identified at the time of collection by the study identity number. Any data included in reports, publications or presented at meetings will be provided in the form of group responses or study identity numbers, such that the participants cannot be identified. Personal and health information (either identifiable or potentially identifiable) about individuals will not be disclosed to any external parties without the individual's consent, unless required by law. The unique study identification code can be broken by the research team if required. It is anticipated that the results of this research study will be published and/or presented in a variety of forums. In any publication and/or presentation, information will be published in a way such that you will not be individually identifiable.

7. How and when will I find out what the results of the research study are?

You have a right to receive feedback about the overall results of this study. You can tell us that you wish to receive feedback by checking the tick box at the end of this form. This feedback will be in the form of a summary of the results. You will receive this feedback after the study is finished.

Results of the study will also be published in newsletters and official website of the School of Optometry and Vision Science, UNSW.

8. What if I want to withdraw from the research study?

You may withdraw at any time. If you do withdraw, you will be asked to complete and sign the 'Withdrawal of Consent Form' which is provided at the end of this document. If you decide to withdraw from the study, we will not collect any more information from you. Any information that we have already collected, however, will be kept in our study records and may be included in the study results.

Alternatively, you can ring the research team and tell them you no longer want to participate.

9. What should I do if I have further questions about my involvement in the research study?

The person you may need to contact will depend on the nature of your query. If you want any further information concerning this project or if you have any problems which may be related to your involvement in the project, you can contact the following member/s of the research team:

Research Team Contact

Name	Prof Fiona Stapleton
Position	Head of the school
Telephone	9385 4375
Email	f.stapleton@unsw.edu.au

What if I have a complaint or any concerns about the research study?

If you have any complaints about any aspect of the project, the way it is being conducted, then you may contact:

Complaints Contact

Position	Human Research Ethics Coordinator
Telephone	+ 61 2 9385 6222
Email	humanethics@unsw.edu.au
HC Reference Number	[INSERT HC reference number]

Consent Form – Participant providing own consent

Declaration by the participant

- I understand I am being asked to provide consent to participate in this research study;
- I have read the Participant Information Sheet, or someone has read it to me in a language that I understand;
- I understand the purposes, study tasks and risks of the research described in the project;
- I understand that the research team will audio/video record the interviews; I agree to be recorded for this purpose;
- I provide my consent for the information collected about me to be used for the purpose of this research study only;
- I have had an opportunity to ask questions and I am satisfied with the answers I have received;
- I freely agree to participate in this research study as described and understand that I am free to withdraw at any time during the project and withdrawal will not affect my relationship with any of the named organisations and/or research team members;
- I understand that I will be given a signed copy of this document to keep;
- I would like to receive a copy of the study results via email or post, I have provided my details below and ask that they be used for this purpose only;

Participant Signature	
Name of Participant (please print)	
Signature of Research Participant	
Date	

Declaration by Researcher*

- I have given a verbal explanation of the research study; its study activities and risks and I believe that the participant has understood that explanation.

Researcher Signature*	
Name of Researcher (please print)	
Signature of Researcher	
Date	

[+]An appropriately qualified member of the research team must provide the explanation of, and information concerning the research study.

Note: All parties signing the consent section must date their own signature.

Form for Withdrawal of Participation

I wish to **WITHDRAW** my consent to participate in the research proposal described above and understand that such withdrawal **WILL NOT** affect my relationship with The University of New South Wales or the School of Optometry and Vision Science.

Participant Signature	
Name of Participant (please print)	
Signature of Research Participant	
Date	

The section for Withdrawal of Participation should be forwarded to:	
CI Name:	Prof Fiona Stapleton
Email:	f.stapleton@unsw.edu.au
Phone:	9385 4375
Postal Address:	Level 3, Rupert Myers building north wing, School of Optometry and Vision Science, UNSW. Kingsford, NSW-2052.

Appendix C

Contact Lens and Lens Case Hygiene

Participant initials: _____ **Participant ID: _____**

Date: _____ **Visit: BL / 1M / 2M / Unsch**

Contact lens Compliance Questionnaire
Please answer following questions on your Contact Lens wear habits (based on you last month habits)

1. CONTACT LENS HISTORY

How long have you been a contact lens wearer for?

☐ 1-3 months ☐ 1-2 years
☐ 4-6 months ☐ 3-5 years
☐ 7-11 months ☐ 6-10 years
☐ More than 10 years

How often do you replace your contact lenses with a new pair?

☐ Daily
☐ Weekly
☐ Every 2 weeks
☐ Monthly
☐ Yearly
☐ Other (*specify* _____)

What is the present brand and name of the lenses you are wearing?
Specify here (if known) _____
How old are your present contact lenses?

_____ days OR _____ weeks OR
_____ months OR _____ years

How many days per week did you wear lenses on average in last month?

☐ Less than once per week
☐ 1-4 days per week
☐ More than 4 days per week

How many hours per day do you wear contact lenses on average in last month?
_____ Hours
At any stage in the previous month, did you have to remove the lenses earlier than usual due to any discomfort?

☐ No

☐ Yes – how many times? _____ (number)
What were the reasons?
☐ Sickness
☐ Discomfort
☐ Other (specify _____)
☐ Your general health changed (specify _____)

Have you used any eye drops to relieve discomfort?
☐ No
☐ Yes (specify_____

2. SOLUTION and LENS CASE HISTORY

Did you use the disinfecting solution last time you cleaned the lens cases?
☐ I don't use disinfecting solution (please specify what you used e.g. tap water/saline)
☐ Sometimes
☐ Every time I reused my lenses

Did you fill lens cases to the mark of the lens cases, when you stored your lens last time?
☐ Yes
☐ No

The last time you removed you lenses, did you rub your lenses before you stored them?
☐ Yes
☐ No
☐ Unsure
☐ Not applicable

The last time you stored your lenses, did you RINSE your lenses before you stored them?
☐ Yes
☐ No
☐ Unsure
☐ Not applicable

The last time you wore lenses, did you RINSE your lenses before you inserted them into your eyes?
☐ Yes If Yes, what did you rinse the lenses with _____
☐ No
☐ Unsure
☐ Not applicable

The last time you wore lenses, did you RUB your lenses before you inserted them into your eyes?
☐ Yes If Yes, what did you rub the lenses with _____
☐ No
☐ Unsure

☐ Not applicable

Did you use any disinfecting solution other than the recommended one?
☐ Yes
☐ No
If Yes, name of the disinfecting solution
_____

Did you top up any remaining solution in your lens cases with the disinfecting solution the last time you stored your lenses?
☐ Yes
☐ No
☐ Unsure
☐ Not applicable

After you took out your contact lenses from your storage case for wear, did you rub the case?
☐ Yes
☐ No
☐ Unsure
What did you rinse your case with (if more than one is applicable, specify ONLY THE LAST ONE used)?
☐ Saline
☐ Water
☐ Disinfecting solution
☐ Other (specify _____)
☐ Unsure
☐ Not applicable

Did you empty your case and leave it to air dry the last time you removed your contact lenses to wear them?
☐ Yes
☐ No
☐ Unsure

Where do you store your lens cases?
☐ Bathroom
☐ Kitchen
☐ Bedroom
☐ Other (specify _____)

3. ENVIRONMENT

The last time you wore lenses did you WASH your hands before you put lenses in your eye?
☐ No
☐ Yes (with soap)
☐ Yes (without soap)
☐ Unsure

The last time you wore lenses did you DRY your hands before you put lenses in your eye?

☐ Yes
☐ No
☐ Unsure

If YES, what did you use to dry your hands?

☐ Bathroom towel
☐ Kitchen roll / paper tissue
☐ Other (specify_____)

Where did you last carry out contact lens insertion and removal?

☐ Bathroom
☐ Kitchen
☐ Bedroom
☐ Other (specify _____)

If the locations for insertion and removal were different, please specify
Insertion: _____
Removal: _____

Did you wear your contact lenses the last time you took a shower?

☐ Yes
☐ No
☐ Unsure

If yes, when did you shower?

☐ Morning
☐ Evening
☐ Both

Do you participate in water activities whilst wearing your contact lenses?

☐ Yes
☐ No

When have you last participated in water activities with your lenses in?
_____ days before or
_____ months before

Where did you last participate in water activities whilst wearing your lenses?

☐ Ocean / Sea / River / Lake
☐ Backyard / private pool
☐ Public pool
☐ Hot tub

Did you wear swimming GOGGLES the last time you participated in water activities whilst wearing your lenses?

☐ Yes
☐ No

Are you a smoker?
☐ Yes
☐ No

If yes, on average, how many cigarettes do you smoke?
_____ per day OR
_____ per week OR
_____ per month

4. EYE DISCOMFORT

During a typical day in the last month, how often did your eyes feel discomfort while wearing your contact lenses?
☐ Never
☐ Rarely
☐ Sometimes
☐ Frequently
☐ Constantly

0_____ 100 No Pain/Discomfort

How intense was the feeling with discomfort?
Explain here_____
_____

Do you have uncomfortable eyes at the end of your wearing time?
☐ Never have it
☐ Not at all intense
☐ Very intense

What was the comfortable wearing time in a day?
_____
____

How long did your eyes feel discomfort at the end of the day while wearing your contact lenses?
_____
____

During a typical day in past one month, how often your eyes feel dry?
☐ Never
☐ Rarely
☐ Sometimes
☐ Frequently
☐ Constantly

0_____ 100 No Dryness/Discomfort

When your eyes felt dry, how intense was the feeling with discomfort?

Explain
here_____
_____

Do you have dry eyes at the end of your wearing time?

☐ Never have it
☐ Not at all intense
☐ Very intense

During a typical day in the past one month, how often did your vision changes between clear and blurry or foggy while wearing your contact lenses?

☐ Never
☐ Rarely
☐ Sometimes
☐ Frequently
☐ Constantly

0_____ 100 No Blur/Difficulty

When your vision was blurry, how noticeable was the changeable, blurry or foggy vision?
Explain
here_____
__

Do you have blurry/foggy eyes at the end of your wearing time?

☐ Never have it
☐ Not at all intense
☐ Very intense

During a typical day in past one month, how often your eyes bother you so much that you wanted to close them?

☐ Never
☐ Rarely
☐ Sometimes
☐ Frequently
☐ Constantly

0_____ 100 Never

How often during a typical day in past one month, did your eyes bother you so much while wearing your contact lenses that you felt as if you needed to stop whatever you were doing and take out your contact lenses?

☐ Never
☐ Less than once a week
☐ Several times a week
☐ Daily
☐ Several times a day

Name of Participant (please print)	
Signature of Research Participant	
Date	

THANK YOU VERY MUCH FOR YOUR HELP WITH OUR STUDY.

PLEASE RETURN THE COMPLETED QUESTIONNAIRE IN THE ENVELOPE PROVIDED

Appendix D

Clinical Trial Notification (CTN)

Australian Government

Department of Health

Therapeutic Goods Administration

Fiona Stapleton
UNSW Australia
Grants Management Office UNSW Australia
Kensington NSW 2052

f.stapleton@unsw.edu.au

CTN SCHEME: ACKNOWLEDGEMENT OF CLINICAL TRIAL NOTIFICATION

Your notification to conduct a clinical trial under the Clinical Trial Notification (CTN) Scheme, pursuant to Schedule 5A of the *Therapeutics Goods Regulations 1990* and/or Schedule 4 of the *Therapeutic Goods (Medical Devices) Regulations 2002* has been received by the Therapeutic Goods Administration (TGA).

Study Sponsor: UNSW Australia (14047)
Protocol Number: SOVS2016-099
Application ID: CT-2016-CTN-04796-1 v1

Details of Therapeutic Goods Notified:

- **Device(s):**

Device Name	Manufacturer	GMDN Code
Sauflon silver barrel lens case	Sauflon Pharmacuiticals	
Sauflon barrel lens case	Sauflon Pharmacuiticals	

Details of Site(s) Notified:

Site Name	State	Site Expected Start Date
School of Optometry and vision Science (SOVS), University of New South Wales	NSW	02/01/2017

In notifying this clinical trial the sponsor of the trial has acknowledged that:

- the sponsor is taking overall responsibility for the trial

PO Box 100 Woden ACT 2606 ABN 40 939 406 804
Phone: 02 6232 8444 Fax: 02 6203 1605 Email: info@tga.gov.au
http://www.tga.gov.au

TGA Health Safety Regulation

- the relevant goods only remain exempt by reason of their use in the clinical trial only for so long as:
    - the approval of the goods for the trial has been given by the sponsor, (if the sponsor is conducting the trial), or by the body or organisation conducting the trial for the sponsor, having regard to the advice of the ethics committee responsible for monitoring the conduct of the trial, on terms no less restrictive than terms advised by that committee
    - the sponsor has not received advice from the ethics committee that is inconsistent with the continuation of the trial
    - the requirements in regulation 12AD of the Therapeutic Goods Regulations 1990 (in the case of therapeutic goods other than medical devices) and regulation 7.5 of the Therapeutic Goods (Medical Devices) Regulations 2002 (in the case of medical devices) are complied with, including that the use of therapeutic goods in the trial must be in accordance with the Guidelines for Good Clinical Practice and the National Statement on the Ethical Conduct in Research Involving Humans published by the National Health and Medical Research Council, as defined in the Therapeutic Goods Regulations
    - the Secretary has not under Item 3 of Schedule 5A of the Therapeutic Goods Regulations (in the case of therapeutic goods other than medical devices) or Item 2.3 in Part 2 of Schedule 4 of the Therapeutic Goods (Medical Device) Regulations 2002 directed that the trial not be conducted on the basis that the Secretary has become aware that to conduct the trial would be contrary to the public interest
- the Secretary can under the *Therapeutic Goods Act 1989* (the Act), require the sponsor to provide specified information or documents relating to any exempt goods
- the Secretary can provide information obtained in response to an authority or the Commonwealth, or a State or Territory that has functions in relation to therapeutic goods or the registration or medical practitioners or pharmacists in the relevant State or Territory
- it is an offence under the Act to fail to provide that information or documents required by the Secretary, or to provide information or documents that are false or misleading in a material particular, to the Secretary
- it is a requirement of the Guidelines on Good Clinical Practice that the sponsor report all serious and unexpected adverse reactions arising from the use of the relevant goods in the trial to the TGA
- it is a serious offence under Commonwealth law to provide information for the purposes of this notification that is false or misleading in a material particular.

Please note that:

i. the Therapeutic Goods Administration has not carried out an assessment of the quality, safety or efficacy of any therapeutic good in relation to this notification.

ii. in the event that the Secretary of the Commonwealth Department of Health becomes aware that to undertake or continue the clinical trial would be contrary to the public interest, the Secretary has the authority to direct that use of the therapeutic good(s) for this clinical trial must cease.

iii. the clinical trial sponsor is still required to comply with any relevant state and territory legislation that might apply in relation to the supply of the therapeutic product(s).

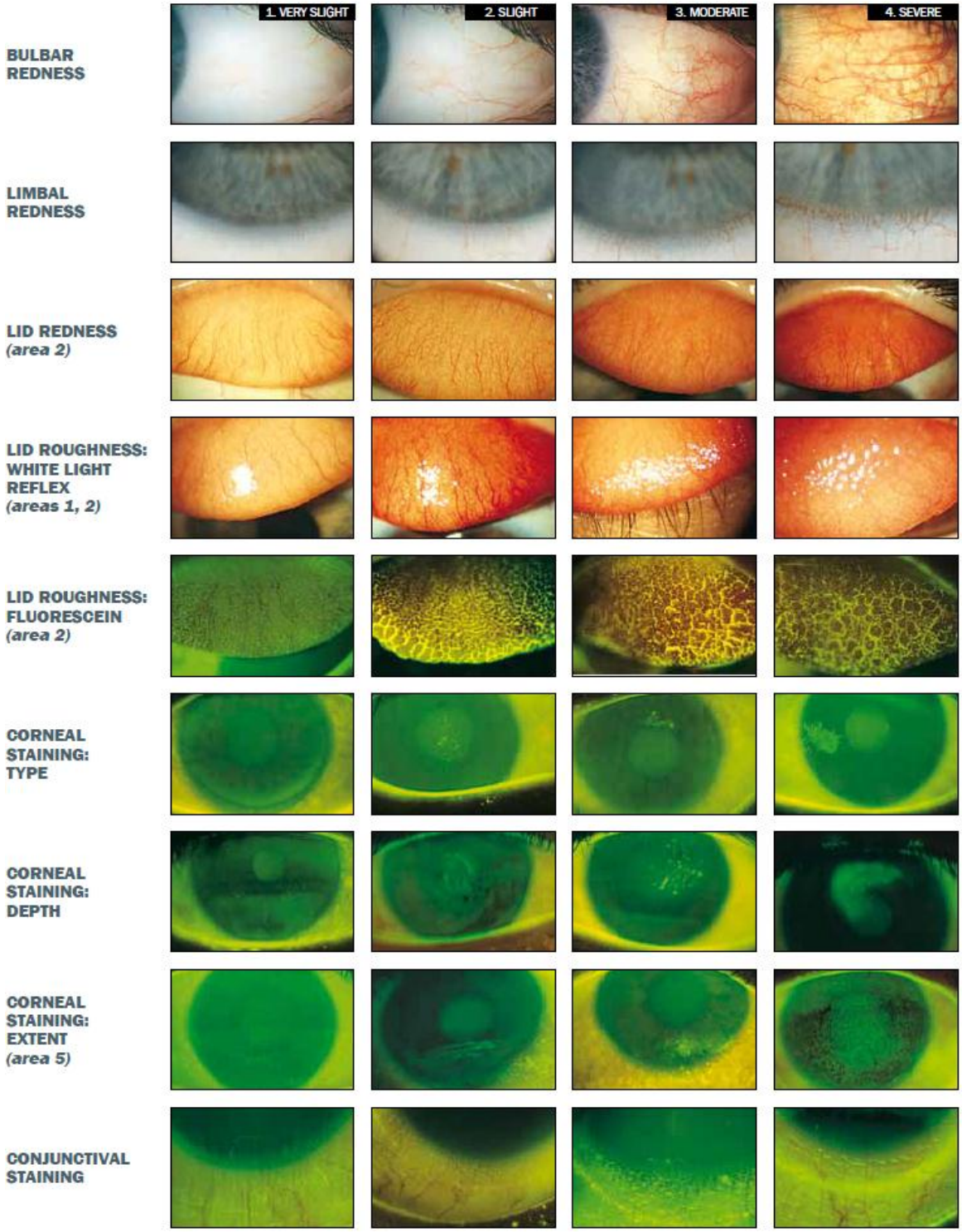
If there are any changes to the trial details notified to the TGA, it would be necessary for the sponsor to update the relevant fields on the online CTN form.

Kind Regards

Clinical Trials Administration
Experimental Products Section

30 November 2016

Appendix E: CCLRU-Brien Holden Vision Institute Grading Scales



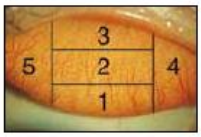
Brien Holden Vision Institute
www.brienholdenvision.org

GRADING SCALES

APPLICATION OF GRADING SCALES

- Patient management is based on how much the normal ocular appearance has changed.
- In general, a rating of slight (grade 2) or less is considered within normal limits (except staining).
- A change of one grade or more at follow up visits is considered clinically significant.

PALPEBRAL CONJUNCTIVAL GRADES



- The palpebral conjunctiva is divided into five areas to grade redness and roughness.
- Areas 1, 2 and 3 are most relevant in contact lens wear.

ADVERSE EFFECTS WITH CONTACT LENSES

CLPC CONTACT LENS PAPILLARY CONJUNCTIVITIS

Inflammation of the upper palpebral conjunctiva

Signs
- Redness
- Enlarged papillae
- Excess mucus

Symptoms
- Itchiness
- Mucus strands
- Lens mislocation
- Intolerance to lenses

INFILTRATES

Accumulation of inflammatory cells in corneal sub-epithelial stroma.
Inset: high magnification view

Signs
- Whitish opacity (focal) or grey haze (diffuse)
- Usually confined to 2-3mm from limbus
- Localized redness

Symptoms
- Asymptomatic or scratchy, foreign body sensation
- Redness, tearing and photophobia possible

CLARE CONTACT LENS ACUTE RED EYE

An acute corneal inflammatory episode associated with sleeping in soft contact lenses

Signs
- Unilateral
- Intense redness
- Infiltrates
- No epithelial break

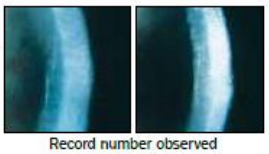
Symptoms
- Wakes with irritation or pain
- Photophobia
- Lacrimation

POLYMEGETHISM

VASCULARIZATION

Vessel extension beyond translucent limbal zone is recorded (mm)

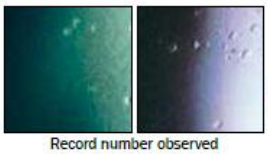
STROMAL STRIAE and FOLDS



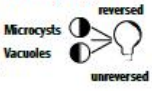
One striae = 5% edema
One fold = 8% edema
(each additional striae or fold indicates 1% more edema)

Record number observed

MICROCYSTS and VACUOLES



Located in epithelium. Identified by side showing brightness.



Record number observed

CORNEAL STAINING GRADES

- Staining assessed immediately after single instillation of fluorescein using cobalt blue light and wratten 12 (yellow) filter over the slit lamp objective.
- The cornea is divided into five areas. The type, extent and depth of staining are graded in each area.

Type
1 Micropunctate
2 Macropunctate
3 Coalescent macropunctate
4 Patch

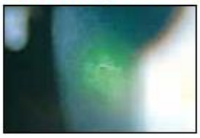
Extent Description
1 ≤5%
2 6-15%
3 16-30%
4 >30%

Depth Description*
1 Superficial epithelium
2 Deep epithelium, delayed stromal glow
3 Immediate localized stromal glow
4 Immediate diffuse stromal glow

* Based on penetration of fluorescein and slit lamp optic section

EROSION

Full thickness epithelial loss over a discrete area

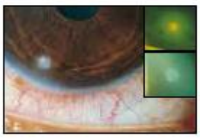


Signs
- No stromal inflammation
- Immediate spread of fluorescein into stroma

Symptoms
- Can be painful
- Photophobia
- Lacrimation

CLPU CONTACT LENS PERIPHERAL ULCER

Round, full thickness epithelial loss with inflamed base, typically in the corneal periphery which results in a scar. Insets: with fluorescein, scar



Signs
- Unilateral, "white spot"
- Localized redness
- Infiltrates
- Post healing scar

Symptoms
- Varies from foreign body sensation to pain
- Lacrimation and photophobia may occur

INFECTED ULCER

Full thickness epithelial loss with stromal necrosis and inflammation, typically central or paracentral

Signs
- Intense redness
- "White patch" (raised edges)
- Infiltrates
- Epithelial and stromal loss
- Anterior chamber flare
- Conjunctival and lid edema

Symptoms
- Pain, photophobia
- Redness, mucoid discharge
- ↓ VA (if over pupil)

Appendix F: Contact Lens dsinfecting Solutions Manufacturers Hygiene Instructions

Contact lens cleaning and disinfecting recommendations:

Stage 1 – Cleaning

Wash, rinse and dry your hands thoroughly before handling you lenses. Place your lens in the palm of your hand and pour on a few drops of your preservative free multipurpose solution. Gently rub both sides of the lens with the tip of your forefinger for 15-20 seconds. Repeat the process with your left lens.

Stage 2 – Rinsing

After cleaning, rinse your lenses with fresh preservative free multipurpose disinfecting solution.

Stage 3 – Disinfecting

Remove the lid from your contact lens case and place your left lens in the left compartment and the right lens in the right compartment of your case, fill the case almost to the mark on the lens case with preservative free multipurpose solution and securely tighten the cap. Leave lenses to soak for at least six hours or overnight.

It is recommended that lenses are re-disinfected after prolonged periods of storage, i.e. seven days or more.

Stage 4 – Wearing

After completion of the disinfecting step the lenses can be removed from the lens case and placed directly on the eye.

Care of the lens case:

After using your contact lens case, thoroughly rinse with your preservative-free multipurpose solution or sterile solution allow to air dry. Do not use soap or detergents. Contact lens case should be kept dry when not in use and replaced frequently as recommended by your contact lens practitioner. Do not rinse contact lenses or lens case with water directly from the tap.

“I am a slow walker, but I never walk back.”

— *Abraham Lincoln*