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EFFECTS OF LONG TERM USE OF CONTACT LENS
DISINFECTING SOLUTIONS ON THE PRESENCE OF
BACTERIA-HARBOURING ANTISEPTIC-RESISTANCE
GENES IN THE CONJUNCTIVAL SAC, EYELID AND ON
THE LENS AND LENS ACCESSORIES OF
ORTHOKERATOLOGY LENS WEARERS

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2016

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Effects of Long Term Use of Contact Lens Disinfecting
Solutions on the Presence of Bacteria-Harboured Antiseptic-
Resistance Genes in the Conjunctival Sac, Eyelid and on the
Lens and Lens Accessories of Orthokeratology Lens Wearers

Shi Guang Sen

A thesis submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

November 2015

CERTIFICATE OF ORIGINALITY

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ABSTRACT

Microbial keratitis (MK) is a rare, but sight-threatening complication associated with contact lens wear. Various factors have been reported as being responsible for contact lens-related MK. Microbial contamination of the contact lens and lens accessories is frequently detected in contact lens wearers and is a known risk factor for MK. *Staphylococcus* species, which are not only commonly isolated from the periorbital region (including conjunctival sac, eye lids, and eyelashes), but also from the contact lenses and lens accessories, are the most common pathogens associated with MK. In published reports, the contamination rates of contact lenses, lens cases, and lens care solutions are high. Contact lenses are considered as vectors capable of transporting potential pathogens contaminating both the lens and lens accessories into the eyes.

To reduce the incidence of ocular infections associated with contact lens wear, the use of contact lens disinfecting and cleaning systems are required. Multipurpose disinfecting solutions (MPS) are essential to inactivate microorganisms on the lenses and lens accessories. Some of the active ingredients of MPS, such as chlorhexidine gluconate (CHX) and benzalkonium chloride (BAK), which have been widely used in hospital and other clinical settings, can cause selective pressure on bacteria which is one of the main reasons for the development of resistance to disinfectants. There is an increased concern about the emergence of disinfectant-resistant microorganisms,

notably in *Staphylococcus*. Antiseptic resistance in staphylococci is attributable to several genes that are mainly plasmid-borne and confer reduced susceptibility to cationic antiseptic agents including dyes, Quaternary ammonium compounds (QACs), and biguanides by coding for efflux pumps, which reduce disinfectant concentration in the cell. The distribution of QAC genes in staphylococci has been investigated not only in clinical isolates, but also in isolates from the general population. In addition, there is evidence that presence of QAC genes may contribute to the emergence of cross-resistance and co-resistance between widely used biocides and antibiotics.

Currently, over 125 million people use contact lenses and lens accessories. More than 90% contact lens wearers are using MPS to clean and disinfect their contact lens and lens accessories. In actual use, especially if not handled properly, MPS are inevitably diluted by water, saline, or other solutions, which will serve to decrease the effects of MPS. Some microorganisms may survive and be exposed to the selective pressure for a prolonged period.

However, it is unknown whether long-term use of MPS can select for ocular pathogens harbouring QAC genes, having increased resistance to several antiseptics, antibiotics, and MPS. The purpose of this research was to provide significant and original contributions to the knowledge base of distribution of QAC genes in *S.*

aureus and coagulase-negative staphylococci (CNS) in selected orthokeratology (ortho-k) lens and spectacle wearers in Hong Kong and observe the trend of resistant organism acquisition over 6 months. It aimed to determine the antimicrobial susceptibility of *S. aureus* and CNS harbouring QAC genes and their association with the presence of QAC genes. It also aimed to investigate the effectiveness of four MPSs for RGP lenses against *S. aureus* and CNS harbouring QAC genes isolated in this research.

This is the first study of the prevalence of QAC gene in staphylococci isolated from ortho-k lens wearers. Samples were collected from several sites in the periorbital region of all subjects, as well as from the lenses and lens accessories of ortho-k lens wearers and from the spectacle frames of controls. Following isolation and identification of bacterial species, all staphylococci were investigated for the presence of QAC genes by polymerase chain reaction. The higher prevalence of QAC genes in staphylococci isolated from ortho-k lens wearers and the gradually increasing prevalence of QAC genes in staphylococci isolated from ortho-k wearers over a 6-month period supported the hypothesis that use of MPS may cause selective pressure to staphylococci to acquire QAC genes. For all staphylococcal isolates harbouring QAC genes, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) to two disinfectants, CHX and BAK, and susceptibility to several antibiotics were determined by broth microdilution method and disc diffusion test, respectively. The results demonstrated that the presence of

QAC genes in staphylococci commonly contributed to higher MIC and MBC to disinfectants and higher resistance rates to antibiotics. Four commonly used MPS for RGP lens were challenged with staphylococci harbouring QAC genes by the stand-alone test and MIC and MBC to each MPS were determined by broth microdilution method. The results demonstrated that the presence of QAC genes in staphylococci significantly decreased their susceptibility to MPS for rigid lenses and viability of some staphylococcal isolates, especially strains harbouring QAC genes, could not be reduced by the required 3-logs after exposure to MPS.

In conclusion, this study has demonstrated that staphylococci harbouring QAC genes are widely distributed in both ortho-k lens and spectacle wearers in Hong Kong, and use of contact lenses increases the likelihood of colonization and contamination with such strains. Use of MPS may contribute to higher carriage rate of gene-positive staphylococci as a result of increased tolerance to disinfectants (including MPS) and antibiotics. Further investigation may be required to investigate the risk of reduced antiseptic and antibiotic susceptibility to MK and its association in the control of MK.

PUBLICATIONS ARISING FROM THE THESIS

Journal Articles

- **Shi GS**, Boost MV, Cho P. Prevalence of antiseptic-resistance genes in staphylococci isolated from orthokeratology lens and spectacle wearers in Hong Kong. *Investigative Ophthalmology and Visual Science*. 2015; 56(5): 3069-3074.
- **Shi GS**, Boost MV, Cho P. Does the presence of QAC genes in staphylococci affect the efficacy of disinfecting solutions used by orthokeratology lens wearers? *The British Journal of Ophthalmology*. 2016 May;100(5):708-12. doi: 10.1136/bjophthalmol-2015-307811. Epub 2015 Dec 30.
- **Shi GS**, Boost MV, Cho P. Prevalence of qac genes increases in staphylococcal isolates from orthokeratology lens wearers over initial six-month period of use. *European Journal of Clinical Microbiology & Infectious Diseases*. 2016 March 18. [Epub ahead of print]
- Cheung SW, Boost M, **Shi GS**, Cho P. Microbial contamination of periorbital tissues and accessories of young children with and without orthokeratology treatment. *Optometry and Vision Science*. 2016 Mar 4. [Epub ahead of print]

Conference Abstracts

- **Shi GS, Cho P, Boost MV.** Prevalence of antiseptic-resistance genes in staphylococci isolated from orthokeratology lens and spectacle wearers in Hong Kong. The 9th Asia Cornea and Contact Lens Conference (ACCLC). 22-23 April 2014, Taiwan.
- **Shi GS, Cho P, Boost MV.** Prevalence of antiseptic-resistance genes in staphylococci isolated from orthokeratology lens and spectacle wearers in Hong Kong. The 19th Congress of Chinese Ophthalmology Society. 17-21 September 2014, Xian, China.
- **Shi GS, Cho P, Boost MV.** Efficacy of multipurpose solutions for rigid gas permeable lenses against staphylococci with disinfectant-resistance genes. The 4th Asia Orthokeratology and Specialty Lens Conference (AOSLC). 13-14 December 2014, Taiwan.
- **Shi GS, Boost MV, Cho P.** Prevalence of antiseptic-resistance genes in staphylococci isolated from orthokeratology lens and spectacle wearers in Hong Kong. The BCLA's 39th Clinical Conference & Exhibition. 29–31 May 2015, Liverpool, UK.

ACKNOWLEDGEMENTS

Many people provided assistance to this project and contributed to the success of this dissertation.

First and foremost, I wish to express my sincere appreciation and gratitude to my supervisor, Prof. Pauline Cho, for her kind guidance, constant encouragement, valuable suggestions, unfailing trust, and generous assistance throughout the course of my study. Prof. Cho taught me a lot on how to perform a research and keep rigorous scientific approach. She offered me a lot of opportunities to learn as much as possible. Her contribution affected my study, my career, and my future.

I would also like to express my sincere thanks to Co-supervisor Dr. Maureen Valerie Boost. Dr. Boost spent many hours and days on this dissertation project. She taught me a lot on how to perform the microbial experimental methods and gave me a lot of guidance on the written of thesis. I am indebted to her for her guidance, never-ending patience and generous support on the whole process of my study and successful completion of this thesis.

I would like to thank colleagues and friends of clinic teams, Peggy Cheung, DeDe

Chan, Cherie Chan, TsuiTsui Lee, Connie Chen, Terry Ng, Angel Wong and Gigi Yee for giving me substantial assistance on subject recruitment and encouragement.

I would like to thank Carol Zhang, Vijaya Chandranna Doddangoudar, and Jeff Ho, who were PHD students and gave me much technical assistance and useful information in this study.

I would like to thank faculty and staff members of school of optometry and Health Technology and Informatics Department, The Hong Kong Polytechnic University for their great assistance during my study periods.

I wish to express my special gratitude to my parents, my wife Dr. Shihui Chen and my beloved daughter, their love and understanding, support and encouragement, give me greatest motion for my staying in Hong Kong for several years.

I acknowledge the postgraduate studentship, The Hong Kong Polytechnic University, Hong Kong for funding this research.

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LIST OF ABBREVIATIONS

agr	Accessory gene regulator
BAK	Benzalkonium chloride
CHX	Chlorhexidine / Chlorhexidine digluconate
CNS	Coagulase negative staphylococci
CTAB	Cetyltriethylammonium bromide
CFU	Colony forming units
EDTA	ethylene diamine tetraacetic acid
EUCAST	European committee on antimicrobial susceptibility testing
FDA	Federal drug administration
FnBP	Fibronectin-binding protein
Ig-binding	immunoglobulin-binding
MAPD	Myristamidopropyl dimethylamine / Aldox
MBC	minimum bactericidal concentrations
MIC	minimum inhibitory concentrations
MK	Microbial keratitis
MLS	Macrolides, Lincosamides, and Streptogramins

MPS	Multipurpose solutions
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin- susceptible <i>Staphylococcus aureus</i>
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHMB	polyhexamethylene biguanide
PIA	Polysaccharide intercellular adhesin
PMMA	Polymethyl methacrylate
PVL	Panton-Valentine leukocidin
PQ-1	Polyquad / Polyquaternium-1
QACs	Quaternary ammonium compounds
QAC gene	Quaternary ammonium resistance gene
RGP lens	Rigid gas permeable contact lenses
sar	Staphylococcal accessory regulator

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Disinfectants based on cationic surface-active agents are extensively used in hospitals, clinics, and other medical settings and play an important role in the control of infectious diseases (Gilbert, *et al.* 2005, Hegstad, *et al.* 2010). The cationic surface-activity of these agents determines their potential to act as a disinfectant on both target and non-target organisms (McBain, *et al.* 2004, Moore, *et al.* 2008). However, the widespread use of these products has led to concern about the increasing incidence of disinfectant-resistant microorganisms worldwide. The relevant genes, *qacA*, *qacB*, *smr*, *qacG*, *qacH*, and *qacJ*, found in many bacteria are in mainly plasmid-borne and confer reduced susceptibility to cationic antiseptic agents including Quaternary ammonium compounds (QACs) (benzalkonium chloride (BAK)), biguanides (chlorhexidine digluconate(CHX)), and dyes (ethidium bromide) (Mitchell, *et al.* 1998, Mayer, *et al.* 2001, Bjorland, *et al.* 2005, Smith, *et al.* 2008, Hegstad, *et al.* 2010, Zhang, *et al.* 2011, Zhang, *et al.* 2012).

Staphylococci species are opportunistic ocular pathogens found in the normal skin and mucosa microbiota, and are commonly isolated from the oral, axillae, inguinal areas and conjunctival sacs of humans (Cogen, *et al.* 2008, Kurzai, *et al.* 2010, Blanco, *et al.* 2013). In the process of proliferation, these bacteria may adapt to changing environments, resulting in the production of molecular changes for proliferation and virulence. Staphylococci are capable of infecting a wide range of plant hosts and animals, including humans (Takeuchi, *et al.* 2005, Beceiro, *et al.* 2013).

In 2004, the number of people using contact lenses and lens accessories was reported to be over 125 million (Barr 2005). Contact lenses and lens accessories are susceptible to microbial contamination (Boost, *et al.* 2005, Yung, *et al.* 2007, Szczotka-Flynn, *et al.* 2010). To reduce the risk of contamination, disinfecting lens care solutions are used to inactivate microorganisms attached to the lenses and lens accessories (Leung, *et al.* 2004, Manuj, *et al.* 2006, Mohammadinia, *et al.* 2012).

Most multipurpose solutions (MPS) comprise QACs or/and biguanides, which have excellent activity against a broad range of microorganisms, including staphylococci and other common ocular pathogens (Miller, *et al.* 2001, Leung, *et al.* 2004, Boost, *et al.* 2010). However, it is unknown whether long term use of these solutions will

select for ocular pathogens with increased resistance to disinfectants, including those used in MPS.

This review gives an overview of the virulence factors of staphylococci and factors affecting colonization and introduces the mechanisms of antiseptic resistance as well as the disinfectant resistant genes.

1.2 Staphylococcal species

Staphylococci are widely dispersed in the environment, they are commonly found colonizing the skin and mucous membranes of up to 80% of humans and also other animals (Nagase, *et al.* 2002). They are also sometimes found in the mammary glands, intestinal, and genitourinary systems, and conjunctival sac of their hosts. In general, the relationship between staphylococci and their hosts is benign or symbiotic. However, if the natural cutaneous barriers are damaged by trauma or other reasons, staphylococci may cause diseases (Murray, *et al.* 2003).

Members of the genus *Staphylococcus* are gram-positive cocci belonging to the

family Micrococcaceae (Murray, *et al.* 2003). Under the microscope, they appear round (cocci) with diameter 0.5 to 1.5µm and occur singly, in pairs, tetrads, irregular clusters, and short chains. They are non-motile and non-spore-forming. Most species demonstrate catalase activity and are facultative anaerobes. Most species survive in the presence of 10% sodium chloride and at the temperature 18-40°C. Their colony sizes range from 1 to 3 mm in diameter within 16-24 hours of incubation.

1.2.1 Classification of *Staphylococcus* species and subspecies

Staphylococci are important nosocomial pathogens. The taxonomy of the genus *Staphylococcus* is still mainly based on the work of Kloos and colleagues (Kloos, *et al.* 1975). Currently, over 40 species and about 20 subspecies have been identified, which can be grouped according to their natural or genomic relationships (Harris, *et al.* 2002). Of these, nine have two subspecies and one has three subspecies.

On the basis of their ability to coagulate plasma, staphylococci are separated into two major groups, namely coagulase positive staphylococci in particular *S. aureus*, and coagulase negative staphylococci (CNS) including *S. epidermidis*, *S. schleiferi* (Baron 1996). *S. aureus* is currently the most common cause of nosocomial

infection leading to increasing concern. *S. aureus* infection is a life threatening disease, especially in elderly and immunocompromised patients (Kang, *et al.* 2011). Formerly, CNS was considered to be very rarely involved in disease. But after the 1980s certain coagulase-negative species including *S. epidermidis*, *S. saprophyticus*, *S. lugdunensis* and *S. schleiferi* have been regarded as opportunistic pathogens (Rogers, *et al.* 2009). The reported incidence of infections due to CNS has been increasing (Rogers, *et al.* 2009). Both *S. aureus* and CNS can cause a wide range of infectious diseases from minor skin and wound infections to more serious diseases such as keratitis, pneumonia, endocarditis, osteomyelitis, sepsis, and bacteremia (Lowy 1998, Rogers, *et al.* 2009).

1.3 Colonization and infections with *S. aureus*

S. aureus is an opportunistic pathogen and the most common pathogen in the coagulase positive staphylococci group. *S. aureus* can cause not only hospital-acquired infections, but also community-acquired infections, such as septic arthritis, osteomyelitis, and skin and softtissue infections (Deurenberg, *et al.* 2008, Dhanoa, *et al.* 2012).

It is well known that 20-30% of humans are long term nasal carriers of *S. aureus*, which usually does not cause any illnesses in these colonized humans (Fitzgerald 2014). Serious staphylococcal infections caused by *S. aureus* commonly occur in groups of people who are at higher risk such as those with chronic illnesses including diabetes, immune-suppression, and cancer (Safdar, *et al.* 2008). According to the Centers for Disease Control and Prevention, the infection rate of serious staphylococcal infections is high in healthcare settings because the immune systems of many of the patients are weak, especially those who have undergone invasive medical procedures (Horan, *et al.* 2008).

S. aureus is widespread in nature, which is mainly isolated from the skin, or nasal mucosa of up to 80% humans. They may also be frequently isolated from the pharynx, perineum, and axillae of these hosts, whilst the gastrointestinal tract, vagina, and conjunctiva are less frequently colonized (Baron 1996, Wertheim, *et al.* 2005, Otto 2010). Wertheim *et al.* (2005) demonstrated that nasal carriage of *S. aureus* is strongly correlated with hand carriage of *S. aureus*. Hands are thought to serve as the main vector to transmit *S. aureus* from surroundings to the nose or other sites, such as to the mouth and conjunctival sac (Wertheim, *et al.* 2005).

About 20-30% of healthy people are asymptomatic carriers of *S. aureus*, and infections in these subjects are commonly caused by the patient's colonizing strain (Wertheim, *et al.* 2005). The colonization rate has been reported to be higher in children (Kluytmans, *et al.* 1997). Three *S. aureus* nasal carriage patterns are recognized in healthy individuals, namely, persistent carriage, intermittent carriage, and non-carriage, respectively. Persistent nasal carriage of *S. aureus* is observed in approximately 20% of the individuals, while approximately 30% carry *S. aureus* transiently, and about 50% are non-carriers (Nagase, *et al.* 2002, Otto 2010).

Persistent carriers commonly have a higher risk of developing a *S. aureus* infection, which might be due to having higher *S. aureus* loads. These individuals are often colonized by the same strain over long time periods (Safdar, *et al.* 2008). However, different strains may be carried in intermittent carriers over time (Chen, *et al.* 2013).

As variation of the colonizing strains is much higher in intermittent carriers than in persistent carriers, this means that the major determinants of persistent carriage in humans are different from those of intermittent carriage. Compared with intermittent carriers and non-carriers, the frequency of colonization of other body sites is higher in persistent *S. aureus* nasal carriers (Wertheim, *et al.* 2005). Persistent carriage rates can be decreased by the improvement of personal hygiene (Wertheim, *et al.* 2005, Ho, *et al.* 2015).

Carriage is the major risk factor for infection, as rates of *S. aureus* infection are three-fold higher in nasal carriers than non-carriers (Nouwen, *et al.* 2006). However, it does not mean that all carriers will eventually acquire infections induced by *S. aureus*. The risk of individual to be infected with *S. aureus* may be associated with several factors, such as their health status, as well as the antibiotic resistance pattern and virulence factors of the *S. aureus* strains (Nouwen, *et al.* 2006).

1.4 Virulence factors of *S. aureus*

S. aureus is the most important staphylococcal pathogen. The main difference between *S. aureus* and other staphylococci is its ability to produce coagulase, but there is little evidence supporting coagulase for its pathogenic predominance. Much work has been done in studying the virulence factors of *S. aureus*. The pathogenic factors of *S. aureus* are generally grouped into three types: the surface-associated virulence factors, the extracellular virulence factors and the regulation of virulence factors (Baron 1996, Bien, *et al.* 2011).

1.4.1 Surface-associated virulence factors

Surface-associated virulence factors, such as wall teichoic acid, surface

immunoglobulin-binding (Ig-binding) protein A and fibrinogen-binding protein (FnBP), play an important role in helping *S. aureus* colonization and avoiding host defense (Baron 1996).

Wall teichoic acid, a surface-exposed staphylococcal polymer produced by *S. aureus*, is a major component of the cell envelope of *S. aureus*. Wall teichoic acid is composed of ribitol phosphate with up to 40 repeating units modified with N-acetylglucosamine and D-alanine (Brown, *et al.* 2013). The significant role of wall teichoic acid in binding to a variety of human cells has been recognised since the 1980s. Several reports demonstrated that wall teichoic acid plays an important role in nasal colonization and provides a site for binding with cationic antimicrobial peptides and proteins (Weidenmaier, *et al.* 2008, Brown, *et al.* 2013).

Surface Ig-binding protein A is found in the cell wall of *S. aureus*. It is a 42kDa surface protein encoded by the *spa* gene and regulated under the control of a two-component system called ArlS-ArlR. It contains five highly homologous extracellular Ig-binding domains, designated domains A, B, C, D, and E. Each domain has the ability to bind proteins from both human and many mammalian species, most notably IgGs. In general, the function of surface Ig-binding protein A

is to capture IgG molecules in the inverted orientation and thereby the bacterial cell is able to avoid being phagocytized by the host immune system (Loughman, *et al.* 2005, O'Seaghdha, *et al.* 2006).

1.4.2 Fibronectin-binding protein

FnBP (Fibronectin-binding protein) adhesins produced by *S. aureus* are members of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family. The effect of FnBPs secreted by *S. aureus* has been well characterized. There are two FnBPs in most *S. aureus* strains, FnBPA and FnBPB. They are encoded by the closely linked genes *fnbA* and *fnbB*, respectively. But some strains of *S. aureus* only express FnBPA. Both proteins are thought to play a significant role in the adherence to host tissues which is necessary for the colonization and the establishment of infection (Brouillette, *et al.* 2003, Wang, *et al.* 2012).

FnBPs are thought to be involved in the infective process, since serum samples from patients with *S. aureus* infections are found to contain anti-FnBP antibodies. In-vitro studies demonstrate that FnBPs are synthesized and expressed during the period of

the exponential growth phase of *S. aureus* and can be degraded by the V8 protease, a staphylococcal protease (McGavin, *et al.* 1997).

1.4.3 Extracellular virulence factors

The wide range of infectious diseases caused by *S. aureus* and their diversity, in part depends on a broad spectrum of extracellular proteins secreted by *S. aureus*, such as enterotoxins (SEA-SEU), hemolysins (α , β , γ), toxic shock syndrome toxin-1 and Panton-Valentine leukocidin (Melles, *et al.* 2006, Qiu, *et al.* 2010). Most *S. aureus* strains produce two or more exoproteins. Each one of these toxins is known to have potent effects on the immune system of their hosts, but many have other additional biological effects. Their main function *in vivo* may be to inhibit host immune responses and enable *S. aureus* to survive inside the host cells (Dinges, *et al.* 2000).

Of these toxins, toxic shock syndrome toxin-1 and the staphylococcal enterotoxins are generally referred to as members of the pyrogenic toxin superantigens group. The pyrogenic toxin superantigens consist of exotoxins secreted by *S. aureus* and *Streptococcus pyogenes* that have been grouped together because they share several crucial biological characteristics. These biological characteristics are pyrogenicity,

superantigenicity, and have the capacity to enhance the lethality of endotoxin (Dinges, *et al.* 2000).

Some pyrogenic toxin superantigens possess additional properties. For example, the staphylococcal enterotoxins are potent emetic agents. Staphylococcal enterotoxins are a family of major serological heat-stable enterotoxins, including the five major classical types (A to E) and some new staphylococcal enterotoxins or SE-like superantigens. Staphylococcal enterotoxins are able to cause a series of problems, such as staphylococcal gastroenteritis, toxic shock-like syndromes, and several allergic and autoimmune diseases (Balaban, *et al.* 2000, Hennekinne, *et al.* 2009).

Toxic shock syndrome toxin-1, a 22KDa exotoxin produced by *S. aureus*, is the key causative toxin of toxic shock syndrome, which is an acute-onset and potentially life-threatening staphylococcal syndrome. Furthermore, both staphylococcal enterotoxins and toxic shock syndrome toxin-1 are able to stimulate the proliferation of T lymphocytes and increase the release of T-cell-derived cytokines (Cullen, *et al.* 1995, Iwatsuki, *et al.* 2006, Lin, *et al.* 2009).

Of the several hemolysins produced by *S. aureus*, α -hemolysin and β -hemolysin are the two most thoroughly characterised of the *S. aureus* cytotoxins (Dinges, *et al.*

2000). α -Hemolysin, a 33kDa monomer, possesses hemolytic, cytolytic, and dermonecrotic activities. Not only erythrocytes are affected by hemolysins. Several other types of human cells are influenced by α -hemolysin, such as lymphocytes, monocytes, and macrophages (Berube, *et al.* 2013). β -hemolysin, an exotoxin with a molecular mass of 35kDa, is secreted by some *S. aureus* strains and causes significant reduction of viability of human polymorphonuclear leukocytes and lymphocytes (Huseby, *et al.* 2007). In general, both α - and β -hemolysins have the ability to cause membrane damage to human monocytes and promote secretion of IL-1 β , IL-6, and TNF- α (Munoz-Planillo, *et al.* 2009).

Panton-Valentine leukocidin (PVL) is a member of the synergohymenotropic toxin family. It constitutes essential components of the virulence mechanisms of *S. aureus*. PVL complex is a two component pore-forming toxin, called F (LukF-PV) (33kDa) and S (LukS-PV) (34kDa). LukS-PV and LukF-PV form a complex and act together. Several studies have shown that LukS-PV first binds to the surface of target cells, which is thought to be a prerequisite step and triggers secondary binding of LukF-PV (Colin, *et al.* 1994, Spaan, *et al.* 2013). LukS-PV and LukF-PV complexes insert into the host-cell membrane to form a pore and start cell lysis (Colin, *et al.* 1994). Several studies have demonstrated that PVL was mainly associated with community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infections

(Vandenesch, *et al.* 2003, Bradley 2005, Maltezou, *et al.* 2006). Bradley (2005) found that PVL was detected in over 80% of isolates from community-acquired staphylococcal necrotizing pneumonia, while none of the hospital-acquired cases were PVL positive. PVL production was designated as a trait of community-associated MRSA and is present in a high percentage of these strains causing severe skin and soft tissue infections in otherwise healthy subjects in the community (Boyle-Vavra, *et al.* 2007).

1.5 Regulation of *S. aureus* virulence factors

S. aureus has the capability of causing a broad range of infections that is probably attributable to the impressive array of extracellular and cell wall-associated virulence determinants expressed by this organism (Bronner, *et al.* 2004). Several two-component regulatory systems, including *agr* (accessory gene regulator) and *sar* (staphylococcal accessory regulator) are capable of coordinating regulation of expression of these virulence factors at the transcriptional level. These systems are sensitive and specific to environmental signals and are composed of two proteins: a sensor histidine kinase and a response regulator.

Several studies have suggested the *agr* system is the most important regulatory system controlling virulence factor expression in *S. aureus*. It serves not only as a repressor of transcription of a number of cell wall associated proteins (e.g. protein A, coagulase, fibronectin binding protein), but is also as an activator of several exoproteins (e.g. toxic shock syndrome toxin-1 and leukotoxins) during the post-exponential growth phase. Expression of several staphylococcal enterotoxins is under the control of the *agr* system, which regulates the expression of staphylococcal enterotoxin genes, such as *seb* and *sec*. The *agr* system works together with DNA-binding proteins, such as the *sar* system, which is another important regulatory system regulating virulence factor expression (Robinson, *et al.* 2005, Cheung, *et al.* 2011). In *S. aureus*, the synthesis of both extracellular (e.g. hemolysins) and cell wall proteins (e.g. FnBP) is activated by the expression of *sar* gene. There are three overlapping transcripts in the *sar* locus, named *sarA* (0.56kb), *sarC* (0.8kb), and *sarB* (1.2kb) (Chien, *et al.* 1999, Bronner, *et al.* 2004, Cheung, *et al.* 2004). Each of these terminates at the same site and encodes SarA protein. SarA, the major *sar* effector molecule, is a pleiotropic regulator that shows negative influence on the synthesis of several virulence factors, such as serine proteinase (*sspA*), surface Ig-binding protein A, and collagen-binding protein (*Cna*). It also controls *sar* target genes in different ways, including direct interaction with or controlling intermediate regulatory molecules such as that of *agr*.

1.6 Coagulase negative staphylococci

CNS has been recognized as an important part of the normal microbiota of the skin and mucous membranes of the humans and other animals (Baron 1996, Rogers, *et al.* 2009). CNS are closely related to *S. aureus*, but are less virulent. They are differentiated from coagulase positive staphylococci by their inability to produce free coagulase. Although, previously thought to rarely cause disease, CNS have been increasingly recognized as causative agents of a wide variety of infections, such as nosocomial bloodstream infections (Giormezis, *et al.* 2014), cardiovascular infections (Chu, *et al.* 2008), urinary tract infection (Kumari, *et al.* 2001), infections of the eye (Green, *et al.* 2008), ear (Shim, *et al.* 2010), and nose (Aral, *et al.* 2003), and the contamination of medical devices, such as indwelling catheters (Kumari, *et al.* 2001), implanted devices (McCann, *et al.* 2008), or contact lenses (Yung, *et al.* 2007). Patients who are immuno-compromised or using intra-venous catheters more frequently suffer from these infections (Rogers, *et al.* 2009, Becker, *et al.* 2014). The specific sites and frequency of infection seem to be related to the site and frequency of normal colonization. For example, *S. capitis* is mainly found on the adult head, *S. cohnii* on the feet, and *S. saprophyticus* in the inguinal and perineal areas (Kloos, *et al.* 1994, Rogers, *et al.* 2009).

The most common CNS species isolated from clinical infections is *S. epidermidis* accounting for approximately 60% of all CNS detected (Vuong, *et al.* 2002). Villari *et al.* (2000) demonstrated that in Naples between 1996 and 1998, 30.4% neonatal infections were attributable to *S. epidermidis*. *S. epidermidis* was also recognized as the main pathogen in meningitis (58.3%), bloodstream infections (39.8%), and skin infections (29.8%) (Villari, *et al.* 2000). In general, nosocomial infectious diseases due to *S. epidermidis* appears to be an increasing problem worldwide (U.S. Department of Health & Human Services Public Health Service 1997) .

S. haemolyticus is recognized as an opportunistic pathogen and the second most frequently encountered CNS species in human infections (Kloos, *et al.* 1994, Barros, *et al.* 2012). Several clinical infectious diseases, including peritonitis, native-valve endocarditis, urinary tract infections, septicaemia, and wound infections are reported to be closely related with this species.

S. lugdunensis is also a commensal bacterium on human skin, but it bears more similarity to *S. aureus* than to *S. epidermidis* or other CNS species with respect to

pathogenicity and virulence (Frank, *et al.* 2008, Rogers, *et al.* 2009). *S. lugdunensis* is commonly isolated from the lower abdomen and extremities, and often occupies different sites from those of *S. aureus*. It not only induces skin and soft tissue infections, but is also the main pathogen of several severe infections with high mortality, such as endocarditis.

S. saprophyticus is an important opportunistic pathogen commonly found on human skin, being more usually isolated from the inguinal and perineal areas (Otto 2004). It is a common cause of human urinary tract infections, especially in young women. *S. saprophyticus* is second only to *Escherichia coli* as the most frequent cause of urinary tract infections in women (Rogers, *et al.* 2009). It has also been regarded as the cause of urinary tract infections in males of all ages and a cause of other sexually transmitted diseases, prostatitis, nephrolithiasis, and wound infections (Otto 2004, Rogers, *et al.* 2009).

The isolation frequencies of the various CNS species in human clinical specimens varies in different areas. For example, generally more than 50% of CNS isolates are identified as *S. epidermidis*. However, in India, only 13.2% of isolates were found to be *S. epidermidis*, but the percentage of *S. haemolyticus* reached 71.9% (Brzychczy-

Wloch, *et al.* 2013). In a Japanese study, Kawamura and co-workers demonstrated a very high percentage of *S. caprae* (10.7%) and a relatively low percentage of *S. lugdunensis* (1.3%), while the frequency of isolation in other studies is quite different (Kawamura, *et al.* 1998, Ehsan, *et al.* 2013).

1.7 Virulence factors of coagulase negative staphylococci

Virulence factors in staphylococci are essentially related to their ability to cause nosocomial infections and some CNS have been shown to display virulence factors contributing to their pathogenicity (Otto 2004).

The pathogenic potential of CNS is thought to be primarily due to their capacity to form biofilms on indwelling medical devices (Otto 2008, Giormezis, *et al.* 2014).

Organisms are able to avoid attacks from antibiotics and the host immune system and survive under the protection of the biofilm. Several factors have received considerable attention with respect to their contribution to biofilm formation. Many CNS are able to produce several lipases, proteases, and other exoenzymes, increasing their persistence in the host and the degradation of host tissue (Otto 2004, Cunha Mde, *et al.* 2006).

1.7.1 Factors involved in biofilm formation

The formation of biofilm commonly comprises two steps: attachment of cells to the biomaterial surface, and accumulation and proliferation of cells to form multilayered cell clusters. There are some virulence factors associated with these processes (Fey, *et al.* 2010).

1.7.1.1 Initial attachment

Several proteins secreted by CNS, especially *S. epidermidis*, are found to be associated with initial adhesion to plastic surfaces. AtlE and Aae, two members of surface-associated protein have been characterized in *S. epidermidis*. AtlE, encoded by *altE*, is reportedly involved in the direct attachment of *S. epidermidis* to polymer surfaces. It was also demonstrated that an *atlE* mutant *S. epidermidis* showed a significant reduction of virulence in a rat model of catheter-related chronic infection. Aae is a novel surface-associated protein with 35kDa having both adhesive and bacteriolytic properties (Heilmann, *et al.* 1996, Gotz 2002, Otto 2004). Other proteins can also mediate the early events of adhesion to uncoated device materials, including biofilm-associated proteins (Bap) and two very large staphylococcal

surface proteins of *S. epidermidis*, SSP-I (280kDa) and its degradation product SSP-2 (250kDa) (Otto 2004, McCann, *et al.* 2008, Fey, *et al.* 2010).

CNS can also attach to host matrix proteins (e.g. fibrinogen, fibronectin, collagen, vitronectin) on host tissues (Baron 1996). Bacterial proteins that bind to host matrix proteins are called MSCRAMMs (Otto 2008). Several MSCRAMMs, such as fibrinogen-binding protein (Fbe) and extracellular matrix-binding protein (Embp), have been well documented (Nilsson, *et al.* 1998, Pei, *et al.* 2001, Christner, *et al.* 2010, Linnes, *et al.* 2013). MSCRAMMs are considered to be important virulence factors as attachment constitutes a crucial step during bacterial colonization (Otto 2004). Often, several MSCRAMMS with overlapping binding capacity are produced in one strain (Gotz 2002, Otto 2004, Fey, *et al.* 2010). Therefore, the loss of one binding protein does not necessarily mean the loss of the capacity to bind to a specific matrix protein. Thus, bacteria prioritize their ability to bind to host matrix proteins, suggesting that binding of these proteins is of enormous importance to the bacteria.

The fibrinogen-binding protein (Fbe), encoded by *fbe* gene, has a molecular mass of 119kDa and is present in most *S. epidermidis* strain (Nilsson, *et al.* 1998, Pei, *et al.*

2001). Antibodies against Fbe can block adhesion to fibrinogen-coated surfaces and implanted catheters, demonstrating that this protein is a major factor mediating adhesion to fibrinogen in *S. epidermidis* (Pei, *et al.* 2001). A second protein found in other *S. epidermidis* strains, which has been called SdrG, has 95% similarity to Fbe and also contributes to fibrinogen binding (Nilsson, *et al.* 1998, Hartford, *et al.* 2001, Fey, *et al.* 2010). SdrG inhibits thrombin-induced clotting of fibrinogen by interfering with the release of fibrinopeptide B. Since fibrinopeptide B functions as a chemotactic molecule, SdrG binding might reduce the influx of phagocytic neutrophils to the infection site. SdrG may therefore have two tasks to help bacterial survival in the host: it can serve as a binding molecule or reduce phagocytic elimination.

Another mechanism of *S. epidermidis* biofilm formation has recently been identified, which is dependent on the expression of a one MDa giant surface protein, the extracellular matrix-binding protein, (Linnes, *et al.* 2013). Christner *et al.* (2010) demonstrated that the extracellular matrix-binding protein acts as a fibronectin-binding protein which is necessary for primary attachment and subsequent biofilm accumulation on fibronectin-conditioned surfaces. Potentially, the extracellular matrix-binding protein may be of additional relevance for the pathogenesis of foreign material associated infections. It was also found that the *embp* gene is

broadly distributed among invasive *S. epidermidis* isolated from blood cultures, prosthetic joint infections, and intraocular infections (Christner, *et al.* 2010, Fey, *et al.* 2010).

Clearly, in order to mediate the initial colonization process during infection, it is a prerequisite for all these proteins not only to interact with the host matrix protein, but also to efficiently bind to the bacterial surface.

1.7.1.2 Cell cluster formation

The second step of biofilm formation comprises the accumulation of bacteria in multi-layered structures. The factors involved in this process differ from those involved in the initial attachment to a surface (Gotz 2002).

The main factor responsible for the formation of cell clusters is an exopolysaccharide of *S. epidermidis*, polysaccharide intercellular adhesin (PIA) (Otto 2008, Agarwal, *et al.* 2010). PIA plays a crucial role in the accumulation phase of biofilm formation and in invasiveness of most staphylococcal species. The main constituent of PIA is

N-acetylglucosamine. As a virulence factor, the importance of PIA has been well demonstrated in animal models of infection (Rupp, *et al.* 1999, Fluckiger, *et al.* 2005).

The intercellular adhesion (*ica*) genes, consisting of *icaA*, *icaD*, *icaB*, and *icaC*, are responsible for the biosynthesis of PIA (Mørseth *et al.* 2003). The *ica* gene locus was first characterized in *S. epidermidis* (Heilmann, *et al.* 1996), but has also been found in *S. aureus* (Cramton, *et al.* 1999) and other CNS, including *S. caprae* (Allignet, *et al.* 2001). In an epidemiological study, *S. epidermidis* strains isolated from patients with nosocomial catheter-related infection more often harbored the *ica* genes and formed biofilms *in vitro* than strains from healthy individuals (Cherifi, *et al.* 2014). The production of PIA seems to be also influenced by the alternative sigma factor, SigB, and by oxygen concentration in *S. epidermidis* (Cramton, *et al.* 2001, Kies, *et al.* 2001). Being a substrate for the synthesis of PIA, glucose, *N*-acetylglucosamine, and glucosamine have been shown to increase PIA production (Otto 2008). Some other factors, such as protein factors, have also been suggested to be involved in the accumulation phase. The 140kDa extracellular accumulation-associated protein enhances the growth of biofilm accumulation on polymer surfaces (Sun, *et al.* 2005). Accumulation-associated protein may be involved in linking PIA to cell surfaces, because PIA was found to be only loosely attached to the cell

surface in mutants lacking this protein (Fitzpatrick, *et al.* 2005, Sun, *et al.* 2005).

Microscopic visualization of the structure of mature biofilms of several bacteria have shown that mature biofilms are not just an amorphous agglomeration of cells, but display a defined structure with channels between mushroom-shaped cell communities (Stanley, *et al.* 2004, Otto 2008). The fluid-filled channels are believed to deliver nutrients to the bacteria. Obviously, regulatory processes are involved in the process of building such a structure. However the mechanism has not yet been determined.

1.7.2 Adaptation to a specific habitat

CNS have to deal with the specific characteristics of the human skin and mucous membranes (Baron 1996, Yao, *et al.* 2006, Otto 2008, Otto 2010). They must adapt to high osmolarity, relatively low pH, mechanical stress, and varying moisture and temperature. A high concentration of blood vessels and sweat glands in the skin means that nutrients are available and consequently, more staphylococci are found in such regions as the scalp, ears, or nares. Most importantly, *S. epidermidis* is known to withstand very high salt concentrations. This is supported by the detection of the

presence of a high number of genes involved in osmoregulation in *S. epidermidis* RP62A (Yao, *et al.* 2006).

The production of adhesion factors can be interpreted as another adaptation to a habitat, as they are needed by the bacteria to efficiently colonize human tissue surfaces (Rogers, *et al.* 2009). Adhesion also constitutes the first step of the infection process, in which the interaction with the host's immune system represents a further factor determining the bacterium's ability to survive. *S. saprophyticus* has a unique adhesion protein, UafA, which allows it to adhere to human ureteral epithelial cells (Park, *et al.* 2010). In addition, *S. saprophyticus* can encode several transport proteins to adjust to osmotic and pH changes and also produces plentiful urease which allows it to proliferate in urine.

As colonizers of the human skin and mucous membranes, CNS have to defend themselves against secreted antibacterial peptides (Otto 2010). During a systemic infection they have to be prepared to encounter other defense mechanisms of innate and adapted immunity. The most important mechanism of CNS to escape from the host's immune system during infection seems to simply hide behind a layer of extracellular matrix (Otto 2004, Otto 2008).

1.7.3 Exoenzymes and toxins

CNS do not produce many factors that would lead to severe sepsis as they do not usually produce molecules with superantigenic activity (Otto 2004). However, some factors produced by CNS, such as lipases, proteases, and phenol-soluble modulins, may contribute to tissue degradation and inflammatory response (Liles, *et al.* 2001, Otto 2004, Saising, *et al.* 2012).

Three lipases, GehC, GehD, and Geh-1, all organized as prepro-enzymes have been shown to be produced by *S. epidermidis* (Rosenstein, *et al.* 2000, Bowden, *et al.* 2002, Sakinc, *et al.* 2005). Lipases from *S. haemolyticus*, *S. hyicus*, *S. warneri*, and *S. xylosus* have also been described (Oh, *et al.* 1999, Rosenstein, *et al.* 2000, Mosbah, *et al.* 2005, Yokoi, *et al.* 2012). Lipases may contribute to virulence by enabling the organisms to survive in the fatty secretions of host skin. In addition, lipase may have a novel role in virulence as GehD of *S. epidermidis* was found to be able to bind to collagen (Rosenstein, *et al.* 2000, Bowden, *et al.* 2002).

There are three different catalytic classes of proteases secreted by staphylococci

species, including metallo-, serine- and cysteine enzymes (Otto 2004, Kantyka, *et al.* 2011). Three proteases have been documented in *S. epidermidis* with different substrate specificities and mechanisms of action: a Zn²⁺-dependent 32kDa metalloprotease, a 41kDa staphopain-like extracellular cysteine protease (Ecp) and a 27kDa serine protease. The activity of the metalloprotease was significantly reduced in a *S. epidermidis agr* mutant strain. Homologues of the metalloprotease were also found in *S. hyicus* and in *S. chromogenes* (Vuong, *et al.* 2000). The cysteine protease is able to degrade several host matrix proteins and components of the immune system *in vitro* (Oleksy, *et al.* 2004). Along with other extracellular proteases, it may contribute to the colonization and infection of human body. The serine protease is preferentially expressed in adherent culture, suggesting a possible role in biofilm formation (Kantyka, *et al.* 2011, Vengadesan, *et al.* 2013).

Recently, a complex of amphiphilic peptides has been described in *S. epidermidis*, called phenol soluble modulins (McCann, *et al.* 2008). Phenol soluble modulins are a complex of (at least) three secreted amphiphilic peptides with inflammatory properties. It causes degranulation, enhanced respiratory burst activity, and inhibition of spontaneous apoptosis in human neutrophils (Liles, *et al.* 2001). Phenol soluble modulins constitute a strong sepsis-inducing factor of CNS. Its discovery has led to reappraisal of the role of CNS in infection (Otto 2004). The production of phenol

soluble modulin is strictly controlled by the expression of *agr*. Low *agr* activity may promote the ability of *S. epidermidis* to colonize catheter material, whilst high *agr* activity increases its invasive capacity. Biofilm formation may be negatively correlated with the *agr* activity in *S. epidermidis*. Phenol soluble modulin may contribute to inhibition of later stages of biofilm formation and lead to biofilm dispersion under *agr* regulation (Klingenberg, *et al.* 2007).

1.7.4 Regulation of virulence factors

Bacteria respond to changing environmental conditions by altering gene expression, which enables them to deal with a variable supply of nutrients, altered oxygen pressure, and the switch from planktonic growth to life in a biofilm (Otto 2004, Kazmierczak, *et al.* 2005). Regulatory systems involved in such basic changes are called global regulators. These regulators also often control the expression of virulence factors. Several global regulators of virulence in CNS are known, including the quorum sensing system *agr*, the *sar* family of transcriptional regulators, and the alternative sigma factor *sigB* (Otto 2004, Kazmierczak, *et al.* 2005).

A quorum sensing system regulates gene expression in response to cell density. The *agr* quorum sensing system was first recognized as a regulator of virulence, but has now been shown to also regulate metabolism (Novick, *et al.* 1999, Batzilla, *et al.* 2006). The *agr* gene generally up-regulates the expression of exoenzymes and toxins and down-regulates the expression of some surface proteins like MSCRAMMS at the onset of post-exponential growth phase (Yao, *et al.* 2006, Otto 2008). Regulating colonization factors, toxins, and exoenzymes by cell density makes sense during an infection, as colonization factors are needed at low cell density at the beginning of an infection, while toxins and degradative factors are needed at high cell density when there is a lack of nutrients and an activated immune response. The *agr* gene constitutes a classic quorum sensing system, which uses auto-regulatory feedback and a peptide-based pheromone (Otto, *et al.* 2001, Otto 2008). The *agr* system is found in every staphylococcal species and the investigation of a deletion mutant of *agr* in *S. epidermidis* showed that it has the role of a regulator of virulence not only in *S. aureus*, but also in CNS species (Vuong, *et al.* 2000, Otto 2008).

The dimeric DNA-binding protein SarA regulates many virulence factors acting as a transcriptional regulator (Chien, *et al.* 1999, Tormo, *et al.* 2005, Cheung, *et al.* 2008). In *S. epidermidis*, SarA is a positive regulator for biofilm formation, modulating *icaADBC* expression and PIA synthesis. The deletion of the *sarA* genes can reduce the

ability to produce PIA/PNAG and biofilm formation *in vitro* (Tormo, *et al.* 2005). It may act directly on the transcription of target genes or via regulation of the *agr* system (Manna, *et al.* 2007). SigB is also present in *S. epidermidis* and controls biofilm formation and expression of lipase and protease production (Kies, *et al.* 2001).

1.8 Resistance mechanisms to various classes of antibiotics

The mechanisms of resistance to antibiotics in staphylococci vary depending on the antibiotic and may be chromosomally and/or plasmid mediated. The emergence of multi-resistant staphylococci can be attributed to the clustering of various transposons carrying different resistance determinants on plasmids, or the chromosome (Woodford 2005).

1.8.1 β -lactam antibiotics

β -lactam antibiotics (including penicillin, cephalosporins, carbapenems, and monobactams) are bactericidal, and act by inhibiting membrane-bound enzymes which are important in the biosynthesis of the cell wall (Kohanski, *et al.* 2010). In *Staphylococci species*, there are three significant mechanisms of resistance to β -lactam antibiotics. The first is to produce β -lactamase (penicillinase) to hydrolyse the

β -lactam ring making the antibiotic inactive. The β -lactamase structural gene, *blaZ*, is responsible for penicillinase production and detected on Tn552-like transposons (Firth, *et al.* 2000). Staphylococcal penicillinase only affects penicillins so activity of cephalosporins and other β -lactams is unchanged. The second mechanism is an intrinsic resistance, involving lowering of the affinity of the penicillin-binding proteins for β -lactams usually involving synthesis of a new penicillin-binding protein (Malouin, *et al.* 1986). This phenomenon, although termed methicillin resistance, leads to reduced resistance to all β -lactams. The third mechanism is tolerance to the bactericidal effect of the β -lactam antibiotics (Tuomanen 1986, Thomas, *et al.* 2013). Tolerance is regarded as an increased resistance to the lethal action of β -lactams and involves cross-resistance to the killing effect of other cell wall synthesis inhibitors.

1.8.2 Tetracycline

Tetracycline is a broad-spectrum agent which inhibits protein synthesis mainly by binding to the 30S ribosomal subunit and preventing the attachment of aminoacyl-tRNA to its acceptor site (Chopra, *et al.* 2001). Four resistant determinants to tetracycline, designated K, L, M, and O, have been detected in staphylococci (Poole 2005). Two resistance mechanisms to tetracycline have been observed in staphylococci (Roberts 1996, Emaneini, *et al.* 2013): (a) Active efflux pumps

encoded by *tetK* and *tetL* genes located on a plasmid. An active tetracycline efflux pump functions as a metal-tetracycline/H⁺ antiporter and leads to resistance to tetracycline. This process is energy-dependent and prevents the accumulation of the antibiotic in whole cells; (b) Ribosomal protection mediated by *tetM* or *tetO* determinants located on either a transposon or the chromosome. Both *TetM* and *TetO* mediate resistance to tetracycline by inhibiting protein synthesis. Presence of *tetM* or *tetO* confers resistance to both tetracycline and minocycline, a lipophilic analog of tetracycline, whereas *tetK* and *tetL* mediate resistance to tetracycline only. It has been reported that most *tetM*-positive isolates also carry *tetK* (Bismuth, *et al.* 1990, Trzcinski, *et al.* 2000).

1.8.3 Macrolides, Lincosamides, and Streptogramins

Macrolide antibiotics (e.g. erythromycin), lincosamides (e.g. clindamycin), and streptogramin antibiotics are chemically distinct, but have a similar mode of action. MLS (Macrolides, Lincosamides, and Streptogramins) antibiotics cause dissociation of the peptidyl-tRNA during elongation stopping protein synthesis by binding to the 50S ribosomal subunit (Kohanski, *et al.* 2010). In staphylococci, resistance to MLS antibiotics is commonly due to the presence of *ermA*, *ermB* and *ermC* genes, present on small multicopy plasmids (Lina, *et al.* 1999). Several other determinants

encoding resistance to MLS antibiotics (such as *msr*, *linA*, *vga*, and *vat*) have been reported in staphylococci (Weisblum 1995, Lina, *et al.* 1999, Chesneau, *et al.* 2005).

1.8.4 Fluoroquinolones

The fluoroquinolones exhibit excellent activity against a broad range of pathogens and include several antibiotics, such as ciprofloxacin, norfloxacin, levofloxacin, gemifloxacin, moxifloxacin, and ofloxacin. They are widely used as effective therapies for urinary tract infection (Linder, *et al.* 2005). They are able to directly disturb bacterial DNA synthesis by inhibiting two bacterial enzymes, DNA gyrase and topoisomerase IV, which have significant roles in inhibiting replication and transcription of DNA. They are able to bind to the complex of DNA and each of these enzymes. Finally, this action will lead to damage of bacterial DNA and death of bacterial cells (Drlica, *et al.* 2008).

Resistance to fluoroquinolones in *Staphylococcus* emerged soon after the commencement of clinical use of these compounds (Acar, *et al.* 1997). Resistance to fluoroquinolones is due to chromosomal mutations in the genes encoding target enzymes or by the induction of a multidrug efflux pump. Mutations in the *gyrA* in

gyrase and *grlA* in topoisomerase IV are the most common sites (Ferrero, *et al.* 1995). These mutations are located at the primary drug targets in staphylococci and lead to decreased susceptibility to fluoroquinolones. NorA, a membrane multidrug efflux pump protein, is capable of transporting ciprofloxacin and several other fluoroquinolones out of the cells and reducing the intracellular concentration of these drugs (Chang, *et al.* 1994, Kaatz, *et al.* 1997, Juárez-Verdayes, *et al.* 2012). Studies have indicated that chromosomal mutations and NorA-mediated resistance could occur alone or together (Kaatz, *et al.* 1997).

1.8.5 Sulfonamides and Trimethoprim

Sulfonamides and trimethoprim act synergistically to affect the same biosynthetic pathway for folate production (Baron 1996). Dihydropteroate synthetase, the target of sulphonamides, catalyzes a key step in the biosynthesis of folic acid. For dihydrofolate reductase, Trimethoprim exhibits a high affinity. It is able to competitively inhibit the reduction of dihydrofolic acid to tetrahydrofolic acid. Resistance to sulfonamide in staphylococci is commonly due to mutations in the *dhps* gene that encodes dihydropteroate synthetase (Alekshun, *et al.* 2007). Chromosomally mediated resistance to trimethoprim occurs via mutations in the *dfrB* gene, which encodes dihydrofolate reductase (Dale, *et al.* 1995). Plasmid-

mediated resistance is mostly due to a unique dihydrofolate reductase (SDHFR), encoded by the *dfrA* gene, which reduces the affinity for trimethoprim. The plasmid pSK639 has been detected in *S. epidermidis*, and subsequently in *S. aureus*, which mediates resistance to trimethoprim via the *dfrA* gene (Apiriridej, *et al.* 1997, Skold 2001, Jensen, *et al.* 2009).

1.9 Disinfectants

Disinfectants are chemical agents that are applied to inanimate objects or surfaces to reduce the number of viable microorganisms to an acceptable level, thereby decreasing the risk of contracting an infection (McDonnell, *et al.* 1999). Although techniques to prevent microbial growth have existed for many years, use of chemical disinfectants to kill or inhibit the growth of microorganisms commenced only 150 years ago (Hugo 1991, Blancou 1995, McDonnell, *et al.* 1999). Once microorganisms were recognized as the cause of most infectious diseases, the science of disinfection rapidly advanced (Powers 1998, Karamanou, *et al.* 2010).

Disinfectants differ from antibiotics, as they lack selective toxicity and target specificity (Fraise 2002). The positive influence of disinfectants in the control of

nosocomial infections has long been recognised (Kawana, *et al.* 2002, Rutala, *et al.* 2004). They are widely used in hospitals (Dettenkofer, *et al.* 2005), clinics (Rutala, *et al.* 2004), and household (Exner, *et al.* 2004) for hand sanitizing, and environmental cleaning to destroy microorganisms that can cause infections. Usually disinfectants cannot sterilize but eliminate a high proportion of microorganisms (usually 99.9%), including bacteria, fungi and virus (Rutala, *et al.* 1999, Rutala, *et al.* 2013).

Chemical disinfectants are categorized into several groups according to their spectrum of activity, mode of action and chemical nature. Alcohols, chlorine compounds, iodine compounds, phenolics, aldehydes (formaldehyde and glutaraldehyde), oxidizing agents (hydrogen peroxide and peracetic acid), QACs, and biguanides are commonly used in hospital for disinfection (McDonnell, *et al.* 1999, Penna, *et al.* 2001, Rutala, *et al.* 2004, Weber, *et al.* 2006, Wijesinghe, *et al.* 2010). Chemical disinfectants are also classified as high-level, intermediate-level, and low-level disinfectants, based on the types of micro-organisms they are able to eliminate (Rutala, *et al.* 2011, Rutala, *et al.* 2013). High-level disinfectants are able to destroy all microorganisms, including bacterial spores. Intermediate-level disinfectants can kill vegetative bacteria and most viruses and fungi, but not bacterial spores. Low-level disinfectants can kill most bacteria, some viruses, and some fungi,

but not resistant bacteria or bacterial spores. High-level disinfectants are commonly used to disinfect heat-sensitive semi-critical items and should never be used on environmental surfaces such as laboratory benches or floors. Only intermediate-level and lower-level disinfectants can be used for disinfection of environmental surfaces or for housekeeping purposes, such as detergent germicides or sanitizers (Rutala 1996, Rutala, *et al.* 2013). Table 1.1 summarises these products, including their active substances and their activity levels. Of these biocides, cationic antimicrobials, including QACs, biguanides, diamidines, and cationic dyes, are commonly used in antiseptics (McDonnell, *et al.* 1999, CDC 2009, Grare, *et al.* 2010).

Table 1.1 Summary of active ingredients in antiseptics and disinfectants.

Disinfectants	Examples	General Use	Activity Level
Alcohols	Isopropanol	Hand sanitizer, rubbing alcohol	Intermediate
	Ethanol	Hand sanitizer, rubbing alcohol	Intermediate
Chlorine compounds	Bleach	Disinfectant, bleaching, water treatment	Intermediate
Iodine compounds	Iodophor	Surface disinfectants	Intermediate
Aldehydes	Formaldehyde	Disinfectant	High to
	Glutaraldehyde	Sterilisation	Intermediate
Oxidizing agents	Hydrogen Peroxide	Disinfectant, contact lens solution	High to
	Peracetic Acid	Surface disinfectants, house use, water treatment,	Intermediate
QACs	BAK, CTAB	Preservative, surface disinfectants	Low
	Polyquaternium-1	Preservative, contact lens solution	
Biguanides	Chlorhexidine	Disinfectant, preservative	Intermediate
	PHMB	Surface disinfectants, water treatment, contact lens solution	Low

QACs: Quaternary ammonium compounds; BAK: benzalkonium chloride; CTAB:

cetyltrimethylammonium bromide; PHMB: polyhexamethylene biguanide

1.10 Cationic antimicrobials

Cationic antimicrobials play an important part in the practice of infection control. They have been widely used for over 50 years as effective agents to kill or inhibit microorganisms (Grare, *et al.* 2010). These compounds belong to a chemically diverse group, but all have a similar mechanism of action. Their mode of action typically involves interaction with the cell membrane by substituting divalent cations (Gilbert, *et al.* 2005, Moore, *et al.* 2008). The exposure to cationic antimicrobials will normally result in interactions with membrane proteins and the protein lipid bilayer, finally leading to the disruption of the cell membrane and lethal leakage of cytoplasm (Moore, *et al.* 2008, Carmona-Ribeiro, *et al.* 2013). In recent years, there has been an increased deployment of these compounds in contact lens care solutions (Boost, *et al.* 2006), in hospital cleaning products (Dettenkofer, *et al.* 2005) and as swimming pools disinfectants (Goeres, *et al.* 2004) due to their relatively low toxicity and broad spectrum of activity.

Many cationic antibacterial agents have been used for surface and topical disinfection or sterilization. In the hospital environment, they have become an indispensable part of infection control practice and help to prevent nosocomial infections (Gilbert, *et al.* 2005, Carmona-Ribeiro, *et al.* 2013). Cationic

antimicrobials, with a range of structures, can be generally classified into four groups according to cationic group numbers in each molecule (Moore, *et al.* 2008). Notable amongst these agents are bisbiguanides (CHX), Quaternary ammonium compounds (QACs) (cetrimide, BAK), and polyhexamethylene biguanide (PHMB) (McDonnell, *et al.* 1999, Gilbert, *et al.* 2005). However, with the long term and widespread use of these agents, there are increased chances of exposure of bacteria to these biocides at sub-lethal concentrations (Russell 2003, McBain, *et al.* 2004). Increased resistance to these antimicrobial agents has been documented worldwide in isolates from humans (Mayer, *et al.* 2001, Zhang, *et al.* 2011), animals (Bjorland, *et al.* 2001, Wong, *et al.* 2013), and the environment (Zhang, *et al.* 2012, Boost, *et al.* 2014).

1.10.1 Quaternary ammonium compounds

QACs are the most commonly used disinfectants preventing the growth and spread of microorganisms. They are membrane active agents belonging to the cationic agents group (McDonnell, *et al.* 1999, Carmona-Ribeiro, *et al.* 2013). Their basic structure is NR_4^+ with positively charged polyatomic ions. R represent alkyl groups, hydrogen atoms, or substituted alkyl groups linked to the central quaternary nitrogen atom and X- represents an anion, such as Cl or Br (Gilbert, *et al.* 2005).

QAC-based disinfectants have been widely used in many clinical settings including hospitals and clinics for a variety of medical purposes and play a major role in the prevention of infectious diseases due to their broad spectrum of antimicrobial action, low toxicity and relative low cost, but they have limited antimicrobial activity against some microorganisms (e.g. spores, some Mycobacteria, and certain viruses) (McDonnell, *et al.* 1999, Ioannou, *et al.* 2007). QACs are also the main active agents of some contact lens care solutions (Boost, *et al.* 2006, Bruinsma, *et al.* 2006). Apart from their excellent antimicrobial efficacy, QACs also show outstanding performance in hard-surface cleaning (McDonnell, *et al.* 1999).

BAK and cetyltrimethylammonium bromide are the most two commonly used QAC agents. Table 1.2 is a summary of structure of these disinfectants.

Cetyltrimethylammonium bromide is a mixture of n-alkyltrimethyl ammonium bromides where the n-alkyl group is between 8 to 18 carbons long, and BAK is a nitrogenous mixture of alkylbenzyl dimethyl ammonium chlorides of various even-numbered alkyl chain lengths.

Table 1.2 Chemical structures of commonly used quaternary ammonium compounds.

Types	Molecular Formula	Chemical structure
Benzalkonium chloride (BAK)	Variable (n=8, 10, 12, 14, 16,18)	
Cetyltrimethylammonium bromide (CTAB)	C ₁₉ H ₄₂ BrN	

In order to avoid the mistakes made with antibiotics with respect to overuse, it is particularly important to understand the mode of action of QACs and bacterial resistance mechanisms to these compounds (Ioannou, *et al.* 2007, Ferreira, *et al.* 2011). The negative charge on the outer surface of bacterial cells can usually be neutralized by divalent cations (e.g. calcium and magnesium) (Gilbert, *et al.* 2005). The cell membrane approximates to a bilayer consisting of proteins and lipids, wherein the proteins will locate on a specific side of the membrane or cross the whole bilayer (integral proteins). The proteins embedded within the cell membrane carry out different functions and play an important role in maintaining the structural integrity of the cell wall as well as metabolic-related functions, such as transport of

materials into the cell, cell membrane biosynthesis, and the transport of extracellular products. Each of these proteins will also help stabilize the lipid bilayer with specific phospholipid and divalent cations (Singer, *et al.* 1972, Navarre, *et al.* 1999, Palsdottir, *et al.* 2004, Ton-That, *et al.* 2004, Gilbert, *et al.* 2005).

Several studies have been conducted on the mechanism of action of QACs (Ioannou, *et al.* 2007, Sandt, *et al.* 2007, Hegstad, *et al.* 2010). The cytoplasmic (inner) membrane of bacteria is considered to be their initial target (Hegstad, *et al.* 2010). Gilbert *et al.* (2005) suggested the mode of action of QACs on the cell membrane of a microorganism as shown in Figure 1.1. Briefly, QAC are thought to progressively adsorb to the relatively anionic bacterial cell membrane and interact with the phospholipids in the membrane. This effect increases gradually with increasing exposure time and concentration of the QAC. Following adsorption, the QAC reacts with the cytoplasmic membrane after penetrating into the cell membrane, which probably causes the disorganisation of the cytoplasmic membrane. Following the eventual lysis of the cell, intracellular materials leak from the fissure of the cell membrane, and proteins and nucleic acids are degraded ultimately causing cell death (Gilbert, *et al.* 2005).

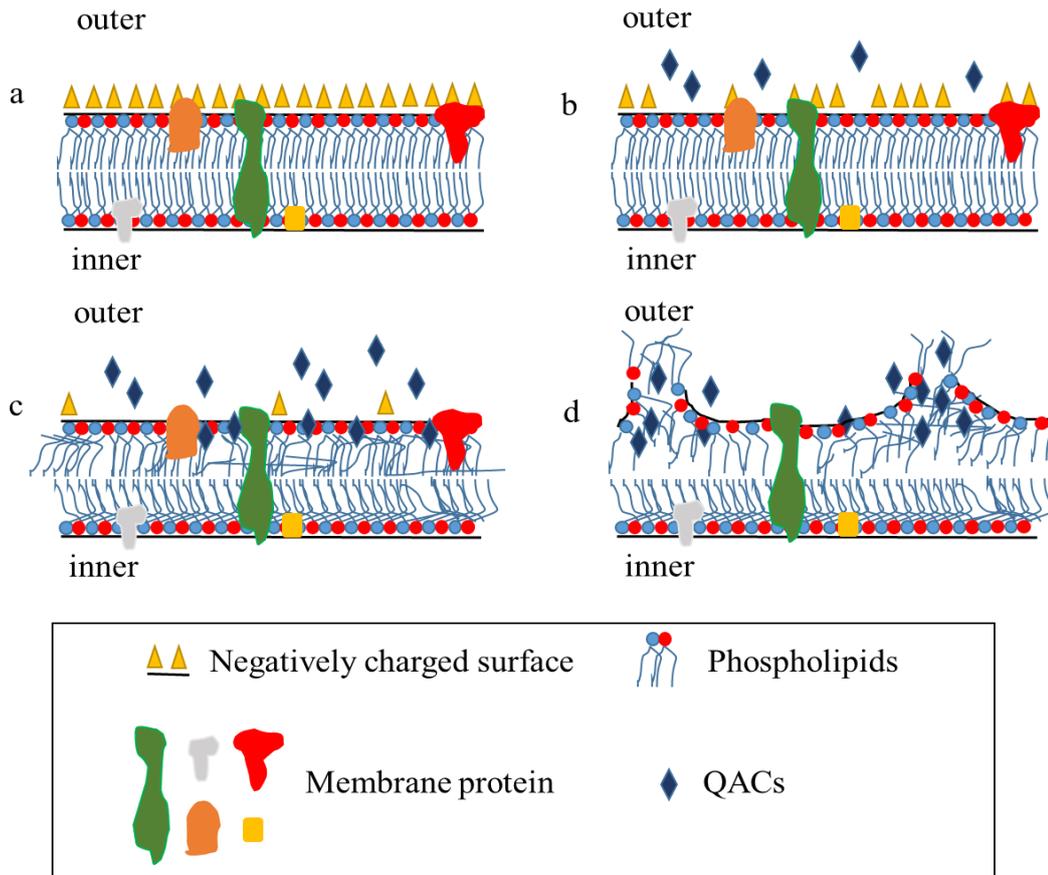


Figure 1.1 Mechanism of action for quaternary ammonium compounds.

a) Normal cell membrane; b) QACs interact with the cell membrane; c) QACs penetrate into the cell membrane; d) Lysis of the cell membrane. (Adapted from Gilbert, *et al.* 2005)

In summary, the mechanism of action of QACs is inducing membrane disorganisation and protoplast lysis through the interaction of their strong positive charge and hydrophobic region with the acidic phospholipid in the cytoplasmic

membrane. Such action results in the damage of the cell membrane structure and leads to cell lysis and cell death.

1.10.2 Biguanides

Biguanides are compounds that contain the $C_2H_5N_7$ component. They are broad-spectrum anti-bacterial biocides, displaying rapid action against both Gram-positive and Gram-negative bacteria (Gilbert, *et al.* 2005, Carmona-Ribeiro, *et al.* 2013).

CHX, a substituted biguanide, has a high degree of antimicrobial activity, low mammalian toxicity, and the ability to bind to the stratum corneum layer of the skin and to mucous membranes (Karpanen, *et al.* 2008, Lokken, *et al.* 2010). Due to its minimal skin irritation properties and persistence on the skin and mucous membranes, CHX is probably the most popular biguanide in disinfectant and preservative products (McDonnell, *et al.* 1999, Gilbert, *et al.* 2005). These unique characteristics make it particularly attractive as an active ingredient in antimicrobial skin preparations.

As with other cationic agents, the activity of CHX relates to its interaction with the cell surface, causing changes in the lipids in the cell membrane (Gilbert, *et al.* 2005, Karpanen, *et al.* 2008). Changes in the integrity of the membrane result in loss of membrane function (McDonnell, *et al.* 1999). The general mechanisms of action of CHX can be summarized as follows (Gilbert, *et al.* 2005):

- Damage to cell membrane by interaction with lipid layer
- Leakage of cellular constituents at low-level exposure
- Precipitation of nucleic acids and proteins at high-level exposure
- Coagulation of intracellular and disruption of enzymatic pathways at high-level exposure

A newer class of biguanide disinfectant is PHMBs, which are mixtures of polymeric biguanides. PHMBs have been widely applied for surface disinfection and water sanitization, particularly as an alternative product to chlorine and bromine. With both a broad spectrum of antimicrobial activity at low concentrations and very low toxicity, PHMBs are also used in contact lens care solution produced by several manufacturers (See Section 1.20.1.1) (Boost, *et al.* 2006).

1.11 Factors affecting disinfectant activity

Several factors may affect the activity of disinfectants, such as concentration, temperature, pH level, duration of exposure, the condition and species of the bacteria, and the presence of organic substances or other compounds.

1.11.1 Concentration

One of the major factors is the effective concentration of the disinfectant, which is an important standard for evaluating the microbial tolerance to disinfectants and has a major impact on clinical practice and application. Most biocides are formulated and used at higher concentration than the effective concentration for direct use on hard surfaces and equipment. In general, if other variables are constant, the higher the concentration of the disinfectant, the stronger its power and the shorter the duration required to kill the microorganisms (McDonnell, *et al.* 1999, Peleg, *et al.* 2000, Russell, *et al.* 2000, Corradini, *et al.* 2003, Tondo, *et al.* 2010, Weaver Jr, *et al.* 2014). The effect of concentration, however, does not apply to iodophors, which consists of iodine and a solubilizing agent (Stiles, *et al.* 1985, Rutala 1996, McDonnell, *et al.* 1999). The antiseptic activity of iodophor is determined by the level of free iodine in an iodophor solution. The amount of free iodine and its antiseptic efficacy decreases with the increasing concentration of iodide in the

iodophor.

1.11.2 Temperature and PH level

The activities of enzymes are closely related with the temperature. Most disinfectants increase their activity when the temperature is increased. However, using heat to increase effectiveness of disinfectants should be carried out carefully, because if the temperature exceeds a certain limit, they may be denatured and some disinfectants, for example chlorine dioxide and peracetic acid, will degrade, (Rutala 1996).

The working pH is an important factor for efficacy of biocides. For example, glutaraldehyde exhibits excellent efficacy at a pH >7 and QACs have optimal efficacy at pH 9 - 10 (Munton, *et al.* 1973, Russell 2004). Changing pH levels can lead to either the disinfectant molecule or the cell surface being altered (Patterson 2009). Increased pH improves activity of some disinfectants, such as QACs and glutaraldehyde, whereas for others, including hypochlorites, phenols and iodine, their effects are increased by a decrease pH (McDonnell, *et al.* 1999).

1.11.3 Duration of exposure

One of the main factors determining the effectiveness of a disinfectant is the length of time it is exposed to the area to be disinfected (Russell 2004, Patterson 2009). As a general rule, the chance of the microorganisms being killed gradually increases with increasing exposure time to disinfectants. To obtain a satisfactory bactericidal effect, the target microorganisms must be exposed to the disinfectant for the necessary contact time, which is also an important standard used to evaluate the potency of the disinfectants. Contact times of disinfectants have been determined and provide useful information for selection of disinfectants in actual application (Rutala, *et al.* 2000, CDC 2009).

1.11.4 Organic material

The antimicrobial activity of disinfectants reduces greatly in the presence of organic matter, such as serum albumin, blood, lipids, or lubricant material (Kawamura-Sato, *et al.* 2008). Organic matter can interact with biocides through a chemical reaction, forming a complex that consumes the biocidal activity, leaving little power to kill microorganisms. Several disinfectants, such as chlorine and QACs, lose their antimicrobial activities due to interaction with organic matter. Additionally, microorganisms may be surrounded by organic material that may act as a physical

barrier to prevent adequate penetration of disinfectant (Kotula, *et al.* 1997, Virto, *et al.* 2005). Kotula *et al.* (1997) demonstrated that available chlorine decreased immediately following exposure to albumin and was determined by the initial concentration of chlorine and the amount of albumin present. After wear, organic material, such as proteins, mucins, and lipids, can be detected on contact lens. Several studies have shown that the effectiveness of most MPS is significantly reduced in the presence of these materials (Boost, *et al.* 2012, Carmona-Ribeiro, *et al.* 2013).

1.11.5 Metal ions

The presence of Metal ions (e.g. K^+ , Ca^{2+} and Mg^{2+}) can have a significant impact on the activity of many disinfectants, such as QACs and biguanides. These disinfectants interact with bacterial cell membrane and destroy the stability of its surface charge. (See Section 1.10.1) The presence of Mg^{2+} or Ca^{2+} may block bacterial adsorption sites which can interfere with the activity of these disinfectants (Crismaru, *et al.* 2011). Crismaru *et al.* (2011) demonstrated that the antimicrobial efficacy of QACs was greatly reduced by addition of 0.1M Ca^{2+} , which may compete with QACs for a place in the membrane. Martin *et al.* (2015) demonstrated that the addition of mono (K^+) or divalent (Ca^{2+}) also inhibited the efficacy of Huwa-San peroxide (a Novel

Silver-Stabilized Hydrogen Peroxide) on killing *E. coli*, but no effect on H₂O₂ mediated killing. The inhibitory effect of divalent Ca²⁺ was much higher than that of monovalent K⁺ at the same concentration (Martin, *et al.* 2015). Hard water contains large amounts of metal ions, especially Ca²⁺ and Mg²⁺ (Sengupta 2013). If they are diluted with tap water, the disinfecting efficacy of MPS may be decreased. Therefore, it is important to disinfect contact lenses properly.

1.11.6 Biofilms

Biofilms are microbial communities in which cells adhere to each other and/or to a surface. An extracellular polymeric substance produced by the bacteria, frequently covers these adherent cells (Prince, *et al.* 2008, Yang, *et al.* 2011). The presence of biofilms on numerous medical devices has been reported (e.g., urinary catheters, central venous catheters, endoscopes, pacemakers, hemodialysis systems, contact lenses, and lens accessories) (Otto 2008, Szczotka-Flynn, *et al.* 2009).

Studies have revealed that although the development of a biofilm differs physiologically between organisms, it can usually be divided into three phases: attachment; maturation stage; and final dispersion (Donlan 2001, Hall-Stoodley, *et*

al. 2002, Chua, *et al.* 2014). In brief, formation of a biofilm begins with a weak, reversible adhesion of free-floating microorganisms to a surface via electrostatic attraction and van der Waals forces. The organisms then anchor themselves more firmly to the surface using cell adhesion structures such as fimbriae and a self-produced matrix of extracellular polymeric substance. Following this, more organisms are able to anchor themselves to the matrix (cell to surface) or earlier colonists (cell to cell). During the subsequent maturation phase, the biofilm grows gradually. During cell division and recruitment, cells are able to contact with each other via quorum sensing mechanisms (Yarwood, *et al.* 2004, de Kievit 2009). At this stage, the biofilm is established and may change only in shape and size. At the final stage of the biofilm life cycle, cells disperse from the biofilm colony, which may be due to nutrient levels or quorum sensing. Dispersal enables biofilm cells to spread and colonize new surfaces.

Microorganisms which form part of a biofilm display increased resistance to disinfectants through several mechanisms, including physical protection against access of disinfectants to the cells within the biofilm, microbial production of degradative enzymes and/or neutralizing chemicals, low rate of growth and metabolism within the biofilm, genotypic variation and/or genetic exchange within the bacteria, and modulation of the microenvironment within the biofilm (e.g., pH)

(McDonnell, *et al.* 1999, Patterson 2009). These microbial communities are tightly attached to the surface and are difficult to remove. Bacteria within biofilms are found to be up to 1,000 times more resistant to antimicrobials than the same bacteria in suspension, which are referred to as planktonic cells (Vickery, *et al.* 2004). All contact lens disinfecting solutions are evaluated according to the Federal Drug Administration (FDA) stand-alone test and need to be able to cause at least a 3-log reduction of viable bacterial cells (planktonic cells) under the recommended disinfection conditions. However, in actual in-use conditions, several bacteria, such as *P. aeruginosa*, *S. marcescens*, and *S. aureus*, can form biofilms on contact lenses and lens cases, and are more resistant to the disinfecting solutions than planktonic cells, which may greatly increase the risk of ocular infections (Szczotka-Flynn, *et al.* 2009).

1.11.7 Innate resistance of microorganisms

When choosing a suitable disinfectant, having a general knowledge of the type of contaminant being targeted is important. One factor which must be considered is that many organisms have naturally high tolerances for certain disinfectants (Rutala 1996, McDonnell, *et al.* 1999). For example, bacterial spores have extremely high

tolerance for many disinfectants, such as QACs and iodine, and require a different disinfectant, stronger concentrations of disinfectant or a longer period of exposure.

The selection of appropriate disinfectants is very complex. Several factors need to be taken into account: type of contaminating microorganisms, degree of contamination, chemical nature of disinfection, concentration and quantity of disinfectant, temperature, contact time, toxicity to the environment and humans, and cost (Rutala 1996, McDonnell, *et al.* 1999, Rutala, *et al.* 2004). Proper use of disinfectant plays a significant role in the control of infectious diseases and preventing the spread of bacteria in hospitals and other clinical settings (Rutala, *et al.* 2004). In addition, the mechanisms of acquired resistance to biocides also need to be considered when assessing the action of biocides (McDonnell, *et al.* 1999).

1.12 Mechanisms of bacterial resistance to disinfectants

With the widespread use of these products, leading to increasing incidence of disinfectant-resistant microorganisms has been a topic of worldwide concern (Sidhu, *et al.* 2001, Pal, *et al.* 2014). The growth and development of bacteria is adversely affected by the presence of biocides and limiting growth conditions. To avoid the

toxic effects of antibiotics and disinfectants, microorganisms have evolved in several different ways to survive in their presence. Bacterial resistance to biocides may allow bacteria to survive in the environment (McDonnell, *et al.* 1999, Poole 2012).

Reports on the resistance to disinfectants have been published worldwide (Sidhu, *et al.* 2002, Langsrud, *et al.* 2003, Zhang, *et al.* 2011). The widespread use and the misuse of disinfectants may lead to selective pressure on bacteria and be the main reason for development of disinfectant resistance (McDonnell, *et al.* 1999, Sidhu, *et al.* 2002). Acquired resistance to disinfectants has been observed, notably in staphylococci and some gram negative bacteria (Kucken, *et al.* 2000, Zhang, *et al.* 2012, Boost, *et al.* 2014).

As with antibiotics, microorganisms can develop acquired resistance to antiseptics and disinfectants by target site alteration and cell wall changes. The genes for these mechanisms may be in the form of plasmids (McDonnell, *et al.* 1999). Target site mutation will commonly lead to over-expression of the gene and production of a special enzyme/protein which regularly results from the emergence of gene mutations. In addition, bacteria may resist disinfectants by altering their membranes, such as changes in outer membrane proteins, which may affect the permeability of

the cell membrane to disinfectants, decrease their uptake and /or increase their discharge (McDonnell, *et al.* 1999, Delcour 2009). Perhaps, the most important mechanism of acquired resistance is the ability to transfer resistance genes between microorganisms (Nikaido 2009, Zhang, *et al.* 2011). Some antimicrobial resistance genes can horizontally transfer within and between species, and so have been found in a variety of bacteria. Resistance to disinfectants may occur by acquisition of a variety of mobile genetic elements, such as plasmids, integrons, and transposons, which contain genes encoding several efflux pumps (Ochman, *et al.* 2000, Dutta, *et al.* 2002). Resistance to disinfectants has been shown to be particularly associated with increased activity of efflux pumps. Bacteria with specific efflux pumps can expel toxic disinfectants out of the cell to decrease their harm to the cell. There are five families of efflux pumps related to antimicrobial resistance described in the following sections.

1.13 Major families of multidrug transporters

Bacteria have developed several ways to survive in the changed environment and resist the harmful effects of disinfectants. Drug efflux pump systems are one of the most important mechanisms of disinfectant resistance, as they allow bacteria to expel a wide range of chemical compounds and toxic substances out of the bacteria via an

energy-dependent mechanism (Poole 2002).

The resistance efflux systems are generally classified as primary or secondary active transports via different energy dependent mechanisms. For the primary active transports, the energy is derived from the hydrolysis of ATP. For the secondary active transports, the energy is generated by the electrochemical proton gradient, named the proton motive force.

Based on the amino acid sequences homology and the similarities in their secondary structure and size, the drug efflux systems have been classified into several transport protein families (Li, *et al.* 2009). The multidrug transporters comprise five superfamilies: The major facilitator superfamily (MFS), the small multidrug resistance family (SMR), the multidrug toxic compound extrusion family (MATE), the resistance nodulation division family (RND) and the ATP binding cassette family (ABC) (Figure 1.2) (Li, *et al.* 2009, Tegos, *et al.* 2011). ABC and MFS are both large families, in contrast to the smaller SMR and RND families (Bay, *et al.* 2009, Pletzer, *et al.* 2014). The fifth family, MATE, was the most recently described (Brown, *et al.* 1999).

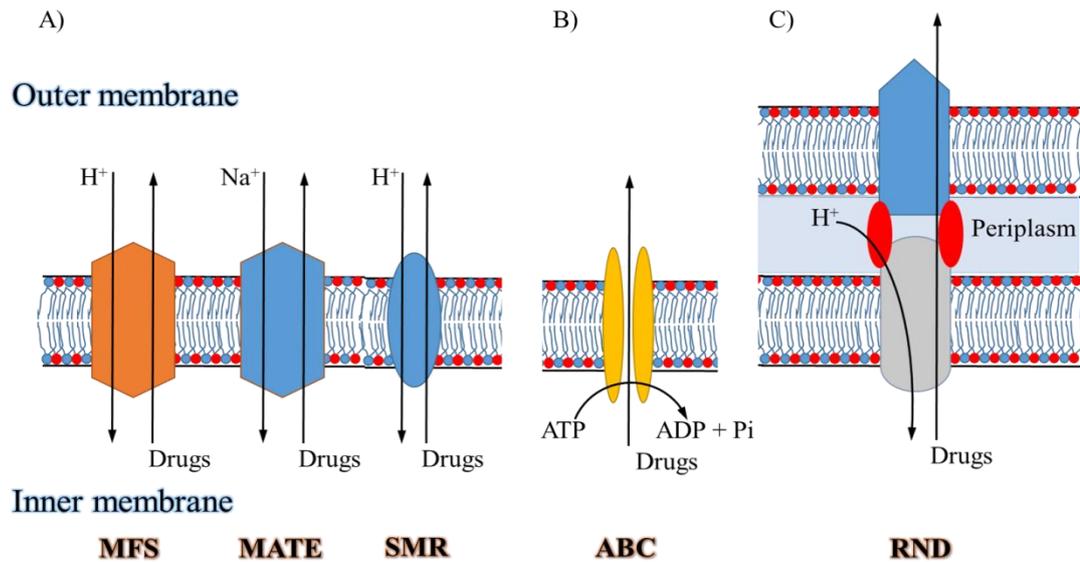


Figure 1.2. Representations of the five families of Multidrug Efflux Systems.

A) MFS, SMR, and MATE are powered by chemiosmotic energy, pumping drugs out of the cell while pumping H^+ or Na^+ into the cell. B) The ATP binding cassette family (ABC) of pumps is powered by ATP. C) RND is a multi-subunit complex spanning the inner and outer membranes in Gram-negative bacteria. (Adapted from Tegos, *et al.* 2011)

1.13.1 Major facilitator superfamily

MFS, also known as the uniporter-symporter-antiporter family, consists of membrane transport proteins which can promote the movement of small molecules across cell membranes following chemiosmotic gradients (Pao, *et al.* 1998).

MFS is the largest group of secondary active transport proteins, being present in Gram-negative and Gram-positive bacteria and higher eukaryotes. The membrane transport proteins of this group are involved in the symport, antiport, or uniport of many substrates (Figure 1.3), such as sugars, phosphate esters, antibiotics and biocides (Pao, *et al.* 1998, Nelson, *et al.* 2008). Initially, MFS were thought to function only in sugar uptake, but later studies revealed that they are also involved in drug resistance (Pao, *et al.* 1998, Reddy, *et al.* 2012).

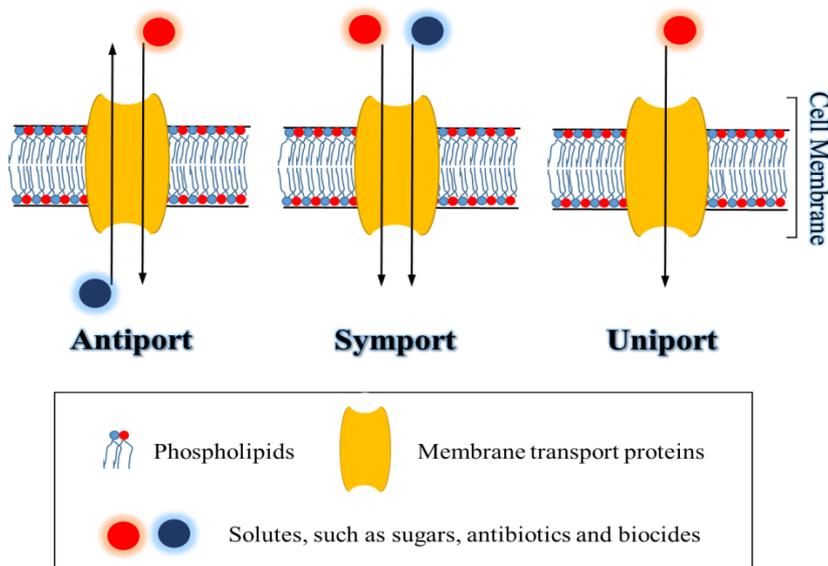


Figure 1.3. Three general classes of membrane transport systems from major facilitator superfamily. (Adapted from Nelson, *et al.* 2008)

So far, more than 70 families have been described within this superfamily, each being involved in the transport of one or more particular type of substrate(s) (Reddy, *et al.* 2012). Among the members of the MFS, two clusters, those having 14-transmembrane segments and those with 12-transmembrane segments, are well documented. *qacA* and *qacB* multidrug efflux proteins of staphylococci have a major role in increasing resistance to a variety of disinfectants and antiseptic compounds such as QACs, diamines, and intercalating dyes. These two efflux pump proteins belong to the 14-transmembrane segments cluster of multidrug transporters (Reddy, *et al.* 2012).

The NorA family multidrug resistance protein was the first to be identified and characterized within a number of chromosomally-encoded drug transporters of the staphylococcal MFS (Ubukata, *et al.* 1989, Noguchi, *et al.* 1999, Hassan, *et al.* 2007, Costa, *et al.* 2013). The NorA protein, a membrane protein with 388 amino acids, is associated with resistance to hydrophobic drugs and belongs to the 12-transmembrane segments cluster of multidrug transporters. NorA is a multidrug transporter and has the ability of transporting a range of dissimilar drugs out of the cell. One study conducted in Japan shows that overexpression of NorA is the likely

cause of significant antiseptic resistance observed in 19 of 98 MRSA isolates collected in 1992 (Noguchi, *et al.* 1999).

1.13.2 Small multidrug resistance family

The SMR family, of proteins which are all approximately 110 amino acids in length, is the smallest group of secondary multidrug drug efflux transporters (Putman, *et al.* 2000, Bay, *et al.* 2008). These drug efflux proteins generally contain four transmembrane segments, which are driven by proton motive force. Based on their structural and energetics characteristics, proteins of the SMR family are different from those of the other four transporter families (Bay, *et al.* 2008).

A large number of annotated protein sequences of the SMR family have been entered into the NCBI protein database. The first gene encoding a multidrug resistance protein of the SMR family was detected in a plasmid present in clinical isolates of *Staphylococcus aureus* (Li, *et al.* 2009). The *smr* transporter of *S. aureus* is the most completely characterized protein of the SMR family, it is able to expel a variety of drugs, cations, dyes and QACs from the cell (Bay, *et al.* 2008). In addition to *smr*, five other *smr*-like genes, *qacE*, *qacEΔ1*, *qacG*, *qacH*, and *qacJ*, have been detected

on plasmids and/or integrons from a variety of disinfectant resistant bacteria (Kazama, *et al.* 1998, Correa, *et al.* 2008, Ye, *et al.* 2012). For example, *qacG*, *qacH* and *qacJ*, encoding multidrug efflux proteins, have recently been identified in *Staphylococcus* isolated from food, animals and from clinical isolates (Heir, *et al.* 1999, Bjorland, *et al.* 2005, Correa, *et al.* 2008, Ye, *et al.* 2012). The protein QacE and QacE Δ 1 of the small multidrug resistance family have been found on integrons in clinical isolates of *K. aerogenes* and *P. aeruginosa* (Kucken, *et al.* 2000).

1.13.3 Resistance nodulation division family

The proteins of the RND family can be detected in many kinds of living organisms, including bacteria, eukaryotes, and archaea, but are mainly distributed in gram-negative bacteria (Paulsen, *et al.* 1996, Routh, *et al.* 2011). The transporter proteins of the RND family are multidrug transporters, which play a significant role in intrinsic and acquired resistance among Gram-negative bacterial strains. Clinically relevant antibiotics (Okusu, *et al.* 1996), dyes (Gugliera, *et al.* 2006), heavy metals (Su, *et al.* 2011), and detergents (Pos 2009) can be driven out of the cell by the efflux pumps of this family. The genes encoding these membrane transporter proteins are mainly found on the chromosome. In recent years, the transporter proteins of the RND division family have also been identified encoding on plasmids

(Li, *et al.* 2009, Yang, *et al.* 2014).

The RND family contains a number of proteins including: AcrB of *E. coli*, *Salmonella*, and *Enterobacter aerogenes* which is a transporter (efflux) protein in the inner membrane (Masi, *et al.* 2003, Pos 2009); TolC of *E. coli* which is an outer membrane protein channel (Andersen 2003); and AcrA of *E. coli* which is a periplasmic accessory protein (Nikaido, *et al.* 2001, Piddock 2006).

1.13.4 ATP binding cassette family

Unlike transporters of the MFS, and the SMR and RND families, the ABC family utilizes hydrolysis of ATP to supply energy, and is not driven by the proton motive force (van Veen, *et al.* 1998, Li, *et al.* 2009). It contains 48 members, which are all integral membrane proteins (Davis 2014). Molecules can be actively transported across the lipid membrane against a concentration gradient, by the energy derived from the hydrolysis of ATP to ADP.

The transporter proteins of the ABC family consist of integral membrane proteins

and cytoplasmic proteins with ATPase (van Veen, *et al.* 1998). They also play an important role in nutrient uptake and expelling toxins and antimicrobial agents out of the cell (Andersen 2003).

The multidrug exporter LmrA (from *Lactococcus lactis*), the first ATP dependent transport system described, confers multidrug resistance in prokaryotes (van Veen, *et al.* 1996). It is encoded by the gene *lmrA* in *L. lactis* and mediates resistance to daunomycin and ethidium bromide. Studies demonstrated that resistance to ethidium bromide in *L. lactis* could be traced to at least two export systems, LmrP and LmrA. They are driven by different energy sources: LmrP depends on the proton motive force, and LmrA is ATP dependent (van Veen, *et al.* 1996, van Veen, *et al.* 2000).

1.13.5 The multidrug toxic compound extrusion family

The MATE family, also known as multi-antimicrobial extrusion protein, is a group of proteins which work as multidrug antiporters for drugs, sodium or protein (Kuroda, *et al.* 2009). In this family, the proteins use the energy of transmembrane sodium electrochemical gradient to pump cationic dyes and fluoroquinolones out of the cell (Putman, *et al.* 2000). The MATE family is the most recently categorized of

the efflux transporter families, being first identified in *Vibrio parahaemolyticus* by Brown and his colleagues (Brown, *et al.* 1999, Kuroda, *et al.* 2009).

Some proteins which now belong to the MATE family were initially considered to be members of the MFS superfamily. However, Brown and his colleagues found that their protein sequences were not homologous with the sequences of MFS proteins, and they were recognized as members of the newly created the MATE family (Brown, *et al.* 1999).

Since the detection of NorM protein in *V. parahaemolyticus* (Brown, *et al.* 1999), several homologues of NorM have been found in other Gram-negative bacteria (Poole 2001, Moreira, *et al.* 2004). The multidrug efflux transporter NorM can expel a variety of antimicrobial agents (e.g. norfloxacin, kanamycin, ethidium bromide) from the cell. NorM protein shows drug/sodium antiporter activity and is the first transporter to function as a sodium-coupled multidrug efflux pump (Morita, *et al.* 2000). NorM is recognized as a prototype of the transporters of the the MATE family and known as "Last of the multidrug transporters". Approximately twenty MATE family transporters from various microorganisms have now been characterized based on their of sequence databases (Kuroda, *et al.* 2009).

1.14 QAC resistance genes

Biocides based on cationic surface-active agents are widely used for disinfection and preventing infection and the transmission of bacteria in hospital, veterinary or industrial environments (Gilbert, *et al.* 2005). Extensive and inadequate use of these disinfectants for disinfection could result in a selective pressure for survival of bacteria and the emergence of resistance genes, thus creating a potentially risk for control of infection in hospitals and other clinical settings (McDonnell, *et al.* 1999).

Several QAC genes that code for resistance to a broad range of disinfectants have been detected in Gram positive and Gram negative bacteria (Kazama, *et al.* 1998, Mayer, *et al.* 2001, Longtin, *et al.* 2011, Zhang, *et al.* 2011, Boost, *et al.* 2014, Zou, *et al.* 2014). The emergence of QAC resistance genes in staphylococcal species have been reported in both human clinical isolates and isolates from the environment (Mayer, *et al.* 2001, Zmantar, *et al.* 2011, Zhang, *et al.* 2012). In general, QAC resistant genes are plasmid-borne and code for the expression of multidrug efflux pumps which are proton motive force dependent. These efflux pumps are membrane-bound transport proteins and can expel a variety of toxic substrates and antimicrobial agents (such as QACs, ethidium bromide and some other cationic biocides) out of the cell (Hegstad, *et al.* 2010, Costa, *et al.* 2013). Microorganisms harbouring these

QAC genes may have the ability to survive in a hostile environment containing disinfectants and antimicrobial agents (Zhang, *et al.* 2011, Zhang, *et al.* 2012). According to their different structures, the gene determinants belong to one of two membrane transportation families: MFS and the SMR family (Putman, *et al.* 2000, Zhang, *et al.* 2012). The location and resistance patterns to biocides of these QAC genes are shown in Table 1.3. *qacA/B* and *smr* are the most frequently reported QAC genes (Longtin, *et al.* 2011, Zhang, *et al.* 2012). The increased resistance to biocides is closely associated with the presence of both *qacA* and *smr* (Zhang, *et al.* 2011).

In 1999, a Japanese study reported the distribution of *qacA/B* or *qacC* (*smr*) genes in clinical isolates of *S. aureus*. The prevalence of *qacA/B* was 10/71 (14%) and *qacC* was 20/71 (28%) in MRSA isolates (Noguchi, *et al.* 1999). Later studies performed by these researchers indicated that the prevalence of MRSA with *qacA/B* had reached 41.6% (372/894) across the Asian region, and in India, *qacC* gene was present in 31.6% of isolates (Noguchi, *et al.* 2005). A study conducted in Hong Kong reported 41.2% (21/51) *S. aureus* isolated from nurses carried the *qacA/B* gene, but the frequency of *smr* was only 11.8% (6/51) (Zhang, *et al.* 2011). In MRSA isolates, the carriage of *qacA/B* was more commonly observed than *smr* (Noguchi, *et al.* 1999, Zhang, *et al.* 2011).

Table 1.3 Staphylococcal QAC genes associated with resistance to biocides.

Gene	Location	resistance biocides	Reference
<i>qacA</i>	plasmid pSK1, pSK105, pSK107, pSK638 and plasmid pSK57 together with resistance genes for β -lactamase and heavy metals	QACs, Eb, Chx, and Diamidines	Lyon, <i>et al.</i> 1984 Gillespie, <i>et al.</i> 1986 Paulsen, <i>et al.</i> 1996 Kupferwasser, <i>et al.</i> 1999 Mitchell, <i>et al.</i> 1999
<i>qacB</i>	plasmid pSK23 together with β -lactam and heavy metal resistance genes	QACs, diamidines and Eb	Paulsen, <i>et al.</i> 1996 Mayer, <i>et al.</i> 2001
<i>smr</i>	large conjugative plasmid with multiple resistance determinants such as pSK41 and small nonconjugative plasmids such as pSK89, pSK108, pST94 and pST827	Some QACs and Eb	Littlejohn, <i>et al.</i> 1991 Heir, <i>et al.</i> 1995 Leelaporn, <i>et al.</i> 1995 Berg, <i>et al.</i> 1998 Heir, <i>et al.</i> 1999 Bjorland, <i>et al.</i> 2001
<i>qacG</i>	plasmid pST94	Some QACs and Eb	Heir, <i>et al.</i> 1999
<i>qacH</i>	plasmid p2H6	Some QACs and Eb	Heir, <i>et al.</i> 1998
<i>qacJ</i>	plasmid pNVH01	Some QACs and Eb	Bjorland, <i>et al.</i> 2003

QACs: quaternary ammonium compounds; Eb: ethidium bromide; CHX: Chlorhexidine

Recently, several novel plasmid-borne genes, *qacG*, *qacH*, *qacJ*, *qacE* and *qacEΔ1*, were detected in staphylococci and some gram negative bacteria associated with infectious diseases. However, the prevalence of these genes are usually lower than that of *qacA/B* (Kazama, *et al.* 1998, Correa, *et al.* 2008, Zhang, *et al.* 2011, Ye, *et al.* 2012).

1.14.1 *qacA/B* gene

The *qacA* gene, which encodes the QacA protein, belongs to the MFS and is harboured on plasmids. Its presence on the plasmid pSK1 of *S. aureus* was first described by (Gillespie, *et al.* 1986). Both the *qacA* and *qacR* genes were identified on the *qacA* locus of plasmid pSK1 (Grkovic, *et al.* 1998). (Table 1.3) *qacR* is found to be located on the site in the *qacA* promoter (IR1, an unusually long operator consisting of 28bp) that overlaps the transcription start site of *qacA*. *qacR*, a divergently transcribed repressor, encodes the QacR protein and regulates the transcription of *qacA*. Lyon *et al.* had previously demonstrated that the *qacA* gene was present on plasmid pSK57 together with other resistance genes for β-lactamase and heavy metals, which suggested that *qacA* is homologous with the antibiotic resistant gene *tet* (Lyon, *et al.* 1984). (Table 1.3) QacA protein has 514 amino acids and mediates resistance to a wide range of substances through the uniport, symport,

and antiport across the cell membrane (Pao, *et al.* 1998, Reddy, *et al.* 2012).

Fluorimetric analyses demonstrated that the *qacA* pump, which has a 14 transmembrane segments configuration, confers resistance to a wide range of structurally diverse hydrophobic drugs via the export of the compound by a proton motive force dependent mechanism (Brown, *et al.* 2001, Hassan, *et al.* 2009). *qacA* has also been detected on other plasmids including pSK105, pSK107, and pSK638 (Kupferwasser, *et al.* 1999). (Table 1.3)

Mitchell and co-workers demonstrated that staphylococcal QacA mediates resistance to more than 30 cationic and lipophilic antimicrobials (e.g. QACs, dyes, and diamidines) belonging to 12 different chemical families. Based on fluorimetric analysis of QacA-mediated export, it was found that export of ethidium bromide could be inhibited by the ionophores nigericin and valinomycin that indicated an electrogenic drug/nH⁺ (n ≥ 2) antiport mechanism (Mitchell, *et al.* 1998). It was also found that the export of ethidium bromide mediated through QacA is both competitively and non-competitively inhibited by monovalent cations, e.g. benzalkonium, and divalent cations, e.g. propamidine, respectively. This suggests that monovalent and bivalent substrates bind at different sites of the QacA protein.

The *qacB* gene, which encodes for the efflux pump QacB, was first detected in clinical isolates of *S. aureus* on plasmid pSK23 again together with resistance genes to β -lactams and heavy metal, and shows high similarity with *qacA* (Paulsen, *et al.* 1996). (Table 1.3) QacB endows high levels of resistance to monovalent cations, such as QACs and dyes (Paulsen, *et al.* 1996, Mitchell, *et al.* 1999). It is important to underline the similarity between *qacB* and *qacA*, which results in it not being possible to distinguish these genes by simple polymerase chain reaction (PCR). There are only seven nucleotide substitution differences between *qacA* and *qacB*, which leads to a single amino acid change at position 323 (Asp in QacA and Ala in QacB). This substitution resulting in the presence of an acidic amino acid in QacA is thought to mediate high levels of resistance to diamidines and biguanidines, which are not found in *qacB* positive isolates (Paulsen, *et al.* 1996, Mayer, *et al.* 2001, Costa, *et al.* 2013).

Using a MIC (minimum inhibitory concentration) method, it was also demonstrated that QacB mediated significantly lower levels of resistance for the diamidines than QacA (Mitchell, *et al.* 1999). The presence of acidic amino acid at position 323 may be involved in substrate binding or recognition which can directly result in increased export of these compounds mediated through the QacA protein (Brown, *et al.* 2001).

1.14.2 *smr* gene

smr, also known as *qacC*, *qacD* or *ebr*, is a plasmid borne gene, often on small plasmids of less than 3kb, that encodes efflux proteins that belong to the SMR family (Leelaporn, *et al.* 1995, Bay, *et al.* 2008). Protein Smr, one of the smallest of the multidrug resistance transporters, plays an important role as a secondary transporter due to its small size. The product, encoded by *smr*, contains 107 amino acids and has four large hydrophobic segments, each having the capability of traversing the cell membrane (Paulsen, *et al.* 1995, Costa, *et al.* 2013). Fuentes and co-workers demonstrated that *smr* identified in *S. epidermidis* conferred resistance to both ethidium bromide and a number of β -lactam antibiotics, which was the first report of the association of a pump of the SMR family with β -lactam antibiotics (Fuentes, *et al.* 2005). Smr protein can mediate resistance to a variety of cationic compounds such as QACs, ethidium bromide and other antimicrobial agents, but its action is somewhat limited compared with QacA (Noguchi, *et al.* 2005).

Some studies have found that *smr*, as well as *qacA* and *qacB*, were more commonly detected in clinical than carriage isolates of staphylococci including *S. aureus* and CNS (Longtin, *et al.* 2011, Zhang, *et al.* 2011). Several studies demonstrated that *smr* gene could be located either on large (>20kb) conjugative plasmids with

multiple resistance determinants such as pSK41 or on small (<3kb) non-conjugative plasmids such as pSK89, pSK108, and pST827 (Littlejohn, *et al.* 1991, Heir, *et al.* 1995, Paulsen, *et al.* 1995, Bjorland, *et al.* 2001). (Table 1.3)

1.14.3 *qacG* gene

qacG gene was first isolated from a staphylococcal plasmid pST94 with 2.3kb in 1999 (Heir, *et al.* 1999). (Table 1.3) The 107 amino acid protein QacG encoded by *qacG*, belongs to the SMR family, and shows 69.2% and 45% similarity with SMR proteins Smr and QacE, respectively. The location of the hydrophobic amino acid in QacG is similar to that of Smr. However, QacG differs from Smr in the position 33 of 107 amino acid of the protein (Heir, *et al.* 1999, Costa, *et al.* 2013).

QacG protein confers resistance to ethidium bromide and QACs via proton dependent efflux. There were only small differences in MIC values to BAK (8–10 mg/L) and ethidium bromide (20–40 mg/L) between isolates harbouring *qacG* gene and *smr* gene, which suggests that the QacG protein might mediate resistance via the same resistance mechanism as the Smr protein (Heir, *et al.* 1999).

1.14.4 *qacH* gene

In 1998, Heir and co-workers isolated a new staphylococcal gene, *qacH*, from a strain of *S. saprophyticus*. The *qacH* gene is harbored on the 2.4kb plasmid (p2H6) (Table 1.3), and mediates resistance to QACs. QacH protein, encoded by *qacH*, contains 107 amino acids and shows strong homology with the SMR family. Further studies revealed that similarity of QacH with Smr and QacG was 78% and 70%, respectively. However, QacH mediated high-level resistance to ethidium bromide, but low-level resistance to proflavine, which is different from similar proteins, Smr and QacG (Heir, *et al.* 1998, Heir, *et al.* 1999, Brown, *et al.* 2001).

1.14.5 *qacJ* gene

qacJ gene present on a 2650bp plasmid pNVH01 in *S. aureus*, *S. intermedius* and *S. simulans* isolated from horses with chronic infections, was first identified in Norway, in 2003 (Bjorland, *et al.* 2003). (Table 1.3) The plasmid pNVH01 was classified as a member of the plasmid pC194 family characterized by rolling circle replication and contained two open reading frames, designated repNVH01 and *qacJ*. *qacJ* encodes a putative protein with 107 amino acid, named QacJ. Homology analysis showed that QacJ was a new member of the sSMR family, and the similarity with other smr protein members was found to be: Smr (72.5%), QacG (82.6%), and QacH (73.4%)

(Bjorland, *et al.* 2003). Compared with Smr, QacG and QacH, QacJ confers a higher resistance level to BAK, but mediates the same resistance level against cetyltrimethylammonium bromide as Smr (Bjorland, *et al.* 2001, Bjorland, *et al.* 2003).

1.15 Link between disinfectant resistance and antibiotic resistance

The development of bacterial resistance has been a major world-wide problem complicating the use of chemotherapeutic agents in the control of infectious diseases (Frimodt-Moller, *et al.* 2007). Furthermore, concern about possible links between antibiotic and disinfectant resistance has been investigated (Lambert 2004). Although there are obvious differences in these antibacterial substances and mechanisms of action between disinfectants and antibiotics, their resistance mechanisms are very similar. For both groups, resistance can be achieved by target mutations, increased target expression, growth in a biofilm, enzymatic inactivation/degradation and, perhaps most importantly, widespread multidrug resistance plasmids (McDonnell, *et al.* 1999, Fraise 2002).

There is increasing concern that sub-lethal concentrations of disinfectants might not

only reduce the effectiveness of disinfectants but also change bacterial susceptibilities to antibiotics. Huet and co-workers demonstrated that multidrug resistance was affected by the activity of an efflux pump, which was over expressed in *S. aureus* after repeated exposures to sub-lethal concentrations of disinfectants in an *in vitro* experiment (Huet, *et al.* 2008). Fraise (2002) proposed that correlation between disinfectant resistance and antibiotic resistance may be due to genetic linkages of their resistance genes that may exist on the same plasmid or be acquired simultaneously. Based on these data, antibiotic resistance was deemed to change with the wide use of disinfectants (Rutala, *et al.* 2000, Fraise 2002, Gasink, *et al.* 2009). However, epidemiological studies about the occurrence of the genes and risk factors for gene carriage are rare. Carriage rate of QAC genes in colonization isolates from nurses and in clinical isolates are higher than in colonization isolates from the general population (Zhang, *et al.* 2011). This supports the hypothesis that increased exposure to disinfectants would increase percentage of QAC genes.

1.16 Use of contact lenses

The history of contact lens is usually thought to begin with the theories of Leonardo da Vinci. He found that the refractive power of the cornea can be neutralized by a water-filled glass hemisphere (Hofstetter, *et al.* 1953, Heitz, *et al.* 1987). However, it

was not until 1887 that August Muller made himself the first pair of contact lenses using polished ground glass (Heitz 1984). After more than a hundred years of development, contact lenses are now widely used to correct refractive errors, or for cosmetic or therapeutic reasons (Roberts, *et al.* 2005, Steinemann, *et al.* 2005).

People choose to wear contact lenses for several reasons. Compared with spectacle wear and refractive surgery, contact lenses offer various benefits, such as providing a wider field of view, providing more comfortable and natural vision, no fogging-up with weather changes, changing the appearance of their eyes, and being practical for sports and other outdoor activities (Walline, *et al.* 2000, Walline, *et al.* 2007, Rah, *et al.* 2010). Additionally, some special corneal conditions or purposes such as keratoconus, irregular astigmatism and myopia progression are typically corrected by rigid gas permeable (RGP) lenses or orthokeratology (ortho-k) lenses (Jupiter, *et al.* 2000, Chen, *et al.* 2013, Chan, *et al.* 2014, Romero-Jiménez, *et al.* 2015).

Globally, there are over 125 million people wearing contact lenses (Barr 2005). In 2012, the global contact lens market was valued at USD 6.81 billion, and is expected to reach USD13.47 billion by 2019 (Transparency-Market-Research. 2014). Hong Kong has a high incidence of myopia, estimated at 144 cases per 1000 primary

school children per annum and significantly increasing with age (Fan, *et al.* 2004, Lam, *et al.* 2012). Therefore, it is expected that there would be a high rate of contact lens use. However, only a few studies have determined usage of different types of contact lenses in Hong Kong (Cheung, *et al.* 2002, Yung, *et al.* 2005, Charm, *et al.* 2010).

1.17 Classification of contact lenses

Contact lens are commonly classified into soft lens and rigid lens according to the characteristics of the material used. Clinically rigid lenses include RGP lenses or ortho-k lenses (Phillips, *et al.* 2007).

1.17.1 Soft contact lenses

Modern soft contact lenses were invented by Otto Wichterle in 1961 (Kopeček 2009). As they are more comfortable than rigid lenses, soft contact lenses are the most popularly used lenses, being used by over 80% of contact lens wearers worldwide (Morgan, *et al.* 2015). Similarly in Hong Kong, the use of soft contact lenses increased from 78% in 1990 to 88% in 2002 (Conway, *et al.* 1990, Cheung, *et al.* 2002). In recent years, the number of soft contact lens wearers has increased

gradually, especially with introduction of silicone hydrogel lenses. Now more than 50% of all soft contact lenses prescribed worldwide are made of silicone hydrogel lens materials (Dumbleton, *et al.* 2010, Morgan, *et al.* 2015).

1.17.2 Rigid lenses

1.17.2.1 Rigid gas permeable contact lenses

Rigid gas permeable contact lenses, also known as RGP lenses, were first introduced in the late 1970s. Most RGP lenses contain silicone, which is oxygen permeable and makes them more flexible and comfortable than the former polymethyl methacrylate (PMMA) lenses (Phillips, *et al.* 2007, Piotrowiak, *et al.* 2014).

Compared with soft contact lenses, better vision, durability, and deposit resistance can be provided by RGP lenses (Bontempo, *et al.* 1997, Szczotka-Flynn, *et al.* 2008). However, the prescribing of RGP lenses worldwide remains low and only accounts for approximately 10% of all lens fits (Morgan, *et al.* 2015). This is primarily because soft lenses can provide instant comfort, while RGP lenses require an adaptation period before they can be worn comfortably. Therefore, RGP lenses are usually only fitted after the failure of soft contact lenses or for some special

conditions, such as keratoconus and irregular astigmatism (Jupiter, *et al.* 2000, Szczotka-Flynn, *et al.* 2008). The use of RGP lenses has been further reduced by the introduction of better soft lenses, such as silicone hydrogel lenses and toric soft lenses (Richdale, *et al.* 2007, Kurna, *et al.* 2010).

1.17.2.2 Orthokeratology

The development of ortho-k therapy originated with the theory of “orthofocus”, in which the cornea was progressively moulded with conventional rigid contact lenses (Jessen 1962). PMMA lens was initially used to flatten the anterior corneal surface. Modern ortho-k emerged in the late 1980s, and differs from traditional ortho-k in lens design and the materials used. It is known as “accelerated orthokeratology” and uses reverse geometry lenses (Lui, *et al.* 2000, Swarbrick 2006, Chen, *et al.* 2009).

Several clinical studies have demonstrated that, for a low to moderate amount of myopia, at least 80% myopic reduction could be achieved by wearing ortho-k lenses (Mountford 1997, Nichols, *et al.* 2000, Cho, *et al.* 2005, Sorbara, *et al.* 2005).

Overnight wear of ortho-k lenses is effective in myopia correction, and up to -6.00D reduction of myopia could be achieved (Swarbrick 2006). In general, ortho-k was

found to be effective in patients with astigmatism less than -1.50D (Cheung, *et al.* 2004, Cheung, *et al.* 2009). More myopic patients with moderate-to-high astigmatism could be included after the introduction of toric ortho-k lenses. Chen and colleagues found that both myopia and astigmatism were significantly decreased by wearing toric ortho-k lenses in myopic children with astigmatism (-1.25D to 3.5D) (Chen, *et al.* 2012, Chen, *et al.* 2013). It has to be stressed that the effects of ortho-k in the reduction of myopia are temporary, and the changes can only be maintained with regular wear of lenses (Soni, *et al.* 2004, Lee, *et al.* 2010).

Several investigators have demonstrated that ortho-k has a significant effect in controlling the progression of axial length elongation. Patients wearing ortho-k lenses showed a slower rate axial elongation of the eyeball compared to those wearing single-vision glasses or soft contact lenses (Cho, *et al.* 2005, Cho, *et al.* 2012, Hiraoka, *et al.* 2012, Charm, *et al.* 2013). Cho *et al.* (2005) found that children with ortho-k lenses showed significantly slower increase in axial length elongation than that of control groups in a 2-year pilot study ($P < 0.001$) (Cho, *et al.* 2005). Even in high myopia, partial reduction ortho-k also effectively slowed myopic progression. After two years of observation, axial length elongation in partial reduction ortho-k-treated children was 63% lower compared with children wearing spectacles (Charm, *et al.* 2013). Children aged under 16 years make up 82% of

ortho-k wearers in Hong Kong (Cho, *et al.* 2003), so practitioners are worried about the potentially serious complications associated with overnight wear of these lenses (Cho, *et al.* 2009). Nevertheless, as there is more understanding of the benefits of orthokeratology, the number of ortho-k lenses wearers is increasing gradually, especially in Asian countries (Cho, *et al.* 2005, Swarbrick 2006).

1.18 Contact lens-associated microbial keratitis

Contact lenses are usually safe and effective for most wearers, but their use can also lead to several problems. Several studies have reported the prevalence of contact lens related complications and some differences were found. These differences can result from several factors, including care systems, contact lens material, wear schedule, and patient-related factors. In 1996, it was reported that the prevalence of contact lens related ocular complications was 39% among 1496 contact lens wearers (Keech, *et al.* 1996). Forister *et al.* (2009) demonstrated that over 50% of the eyes suffered from at least one complication associated with contact lens wear. A large scale study (involving 23068 patients) was performed in Japan to investigate the incidence of corneal complications associated with contact lens wear (Hamano, *et al.* 1994). Compared with other lenses, the highest rate of corneal complications was observed in PMMA lenses (15.8%), and the lowest for daily disposable lenses (2.5%). Rigid

lenses were found to have a lower prevalence of complications than soft contact lenses (Forister, *et al.* 2009, Lee, *et al.* 2012).

Some complications are not serious, such as allergic conjunctivitis, giant papillary conjunctivitis, peripheral infiltrates, corneal staining, neovascularization, but others, such as corneal abrasions and microbial keratitis (MK), can have serious consequences, possibly leading to permanent visual loss (Beljan, *et al.* 2013).

Wearing of contact lenses has been identified as a significant risk factor for MK and was associated with 22% to 52% of MK (Erie, *et al.* 1993, Green, *et al.* 2008, Al-Yousuf 2009, Musa, *et al.* 2010).

1.18.1 Annual incidence of contact lens-associated microbial keratitis

Table 1.4 summarizes the incidence of contact lens-associated MK according to the findings of several published reports. Incidence of contact lens-associated MK varies considerably between studies (Poggio, *et al.* 1989, Nilsson, *et al.* 1994, Cheng, *et al.* 1999, Seal, *et al.* 1999, Lam, *et al.* 2002, Morgan, *et al.* 2005, Dart, *et al.* 2008, Stapleton, *et al.* 2008). Variation may be due to differences in study design, location, definition of MK, and the power to detect differences.

Infections are thought to be more frequent in soft lens users than in rigid lenses wearers. Compared with daily-wear of soft lenses, extended wear also exhibits a higher risk of MK (Morgan, et al. 2005, Stapleton, et al. 2008).

Table 1.4 Annual incidence of contact lens-associated microbial keratitis.

Author	Cases	Location	Incidence per 10000 wearers		
			Daily wear soft lenses	Extended wear lenses	Daily wear rigid gas-permeable lenses
Poggio, <i>et al.</i> 1989	195	United States	4.1	20.9	--
Nilsson, <i>et al.</i> 1994	26	Sweden	2.2	13.3	1.5
Cheng, <i>et al.</i> 1999	92	Netherlands	3.5	20	1.1
Seal, <i>et al.</i> 1999	27	Western Scotland	2.7	--	0.8
Lam, <i>et al.</i> 2002	59	Hong Kong	3.1	9.3	0.4
Morgan, <i>et al.</i> 2005	38	UK	6.4 (Severe keratitis)	96.4 (Severe keratitis)	2.9 (Severe keratitis)
Stapleton, <i>et al.</i> 2008	285	Australia	1.9	19.5	1.2
Dart, <i>et al.</i> 2008	367	UK	3.5	20	1.1

MK associated with orthokeratology lens wear was first reported in 2001 (Chen, *et al.* 2001). Since then, an increasing number of relevant cases were reported worldwide, mainly in China and Taiwan, and more than 50% of reported infections occurred in 2001 (Watt, *et al.* 2005, Watt, *et al.* 2007). In 2013, Bullimore and colleagues reported the estimated incidence of MK associated with overnight ortho-k wear was 7.7 per 10000 wearers-year (Bullimore, *et al.* 2013), which is similar to that for extended wear of soft contact lenses but higher than that for daily wear of rigid lenses (Table 1.4).

1.18.2 Risk factors for contact lens-associated microbial keratitis

There are a variety of specific risk factors, which can promote the development of MK in contact lens wearers. To reduce the risk of infection, several studies have been carried out to investigate these risk factors (Willcox, *et al.* 2001, Weissman, *et al.* 2002, Booranapong, *et al.* 2012).

The intact corneal epithelium presents a substantial barrier to infection. Before a microbe can establish an infection, it must adhere to its target. Any disruption of this barrier will increase the risk of MK (Alarcon, *et al.* 2011). Minor epithelial erosions

are commonly seen in all contact lens users. Contact lens wear can cause corneal epithelial defects due to indentation or abrasion from the lens edge or foreign bodies, which will create opportunities for adherence of pathogens (Forister, *et al.* 2009).

It is well known that contact lens induced hypoxia alters the physiology and metabolic activities of the cornea and results in multiple changes in the epithelium, stroma, and endothelium. Extended periods of low oxygen supply diminish corneal sensation and increase risk of keratitis (Bonanno 2001, Chhabra, *et al.* 2009, Leung, *et al.* 2011). Ren *et al.* (1999) demonstrated that hypoxia at the corneal surface led to metabolic compromise of the epithelium, rendering it less resistant to microbial infection. Compared with hydrogels, silicone hydrogel lenses have greater oxygen permeability that may result in a lower risk of infectious keratitis (Ren, *et al.* 1999). With the availability of more permeable lenses, contact lens induced hypoxia has been greatly reduced.

As mentioned above, extended wear or over-wear use of contact lenses is well known as a major risk factor for MK (Morgan, *et al.* 2005, Stapleton, *et al.* 2008). Extended wear of contact lenses disturbs the corneal metabolism that compromises corneal defence mechanisms making it more susceptible to infection. Lam *et al.*

(2002) demonstrated that the risk of MK was increased three times with extended wear of contact lenses compared to daily wear (Lam, *et al.* 2002). Silicone hydrogel lenses allow up to five times more oxygen to pass through to the cornea than conventional hydrogel lenses (Compan, *et al.* 2002, Lopez-Aleman, *et al.* 2006). In several large-scale epidemiological studies, it was found that the risk of infection for 30 nights of silicone hydrogel use was equivalent to six nights of hydrogel extended wear (Stapleton, *et al.* 2013). Overall, overnight lens wear involves a greater risk of MK than daily lens wear (Eltis 2011, Stapleton, *et al.* 2013).

Poor compliance with lens hygiene is another major risk factor in contact lens-associated MK (Eltis 2011). Several studies have been performed to evaluate compliance and have reported that up to 91% of contact lens wearers show some degree of non-compliance in the process of handling their lenses (Claydon, *et al.* 1994, de Oliveira, *et al.* 2003, Yung, *et al.* 2007, Cho, *et al.* 2009, Bui, *et al.* 2010, Wu, *et al.* 2010, Wu, *et al.* 2011). Morgan (2008) summarized a number of studies of compliance and other factors with close relationship with MK and concluded that improper case care resulted in a higher risk of infection (Morgan 2008).

Microorganisms may have an increased chance to colonize improperly handled lenses and lens accessories and the contact lens may then act as a vector, transferring pathogens to the ocular surface. Several studies demonstrated that a lower rate of

contamination of contact lenses and lens accessories was observed among patients with good compliance (Boost, *et al.* 2005, Wu, *et al.* 2015, Wu, *et al.* 2015). It was found that subjects' awareness of the importance of compliance can be improved by regular education (Yung, *et al.* 2007, Cho, *et al.* 2009).

Clinical reports have suggested additional risk factors for development of MK in contact lens wearers, such as presence of chronic disease, smoking, male gender, swimming with contact lenses, and rinsing contact lenses and/or lens case with tap water (Weissman, *et al.* 2002, Morgan, *et al.* 2005, Booranapong, *et al.* 2012, Stapleton, *et al.* 2012, Wu, *et al.* 2015).

1.18.3 Microbial keratitis inducing pathogens

Many studies have demonstrated that a variety of microorganisms are associated with contact lens-associated MK, including bacteria, fungi and amoeba (Willcox, *et al.* 2001, Green, *et al.* 2008). Contact lens wearers are more susceptible to bacterial infection and many infections are caused by normal microbiota on the ocular surfaces (Fleiszig, *et al.* 2010). These normal microbiota protect the eye from more virulent microorganisms but they can also act as opportunistic pathogens and induce

MK in those patients in whom the cornea or immune system is compromised (Baron 1996, Fleiszig, *et al.* 2010).

The most common bacterial pathogens involved in contact lens associated MK are *P. aeruginosa* and *Staphylococcus*. Others occasionally reported include *Streptococcus*, *Bacillus*, *Enterobacter*, *Acinetobacter*, and *Serratia*. In some cases, positive corneal cultures contain more than one microorganism (Fleiszig, *et al.* 2010, Eltis 2011). In addition, an increasing number of *Acanthamoeba* keratitis cases have been reported in contact lens wearers in recent years. Several factors and activities are thought to increase the risk of *Acanthamoeba* keratitis, including rinsing contact lenses with contaminated tap water, using homemade solutions to store contact lenses, and swimming or showering with contact lenses (Thebpatiphat, *et al.* 2007, Boost, *et al.* 2008, Ku, *et al.* 2009, Verani, *et al.* 2009).

1.19 Contamination of contact lenses and lens accessories

Keratitis inducing pathogens are not only isolated from the corneas but also from contact lenses and lens accessories used by patients (Yung, *et al.* 2007, Boost, *et al.* 2008, Konda, *et al.* 2014, Wu, *et al.* 2015). Contact lenses and lens accessories are

susceptible to contamination with microorganisms. In published reports, the contamination rates of contact lenses and lens accessories varied greatly (Table 1.5). Reports of the contamination rates of contact lenses, lens cases and lens care solutions ranged from 9% to 84%, 25% to 87% and 11% to 63%, respectively (Devonshire, *et al.* 1993, Hart, *et al.* 1993, Gray, *et al.* 1995, Lipener, *et al.* 1995, Rosenthal, *et al.* 1995, Midelfart, *et al.* 1996, Velasco, *et al.* 1996, Sweeney, *et al.* 1999, Boost, *et al.* 2005, Yung, *et al.* 2007, Cho, *et al.* 2009, Willcox, *et al.* 2010).

1.19.1 Contamination of contact lenses

Several studies have been performed on evaluating bacterial contamination of contact lenses among asymptomatic contact lens wearers. There is a wide variation, ranging from 9% to 84%, in contamination rates of contact lens across these studies (Table 1.5). This variation results from several factors, including the differences between subjects, sampling techniques, lens materials, lens care systems, modes of wear and geographical locations. In several studies, an unusually high percentage (over 80%) contamination rate of contact lenses was observed. This may be because the lenses were sampled immediately after one day of wear (Velasco, *et al.* 1996).

Table 1.5. Reports on contamination rates of contact lenses, lens cases and solutions.

Author	Country	Number of Subjects	Contact Lenses	Cases	Solutions
Devonshire, <i>et al.</i> 1993	Scotland	46 rigid & 132 soft contact lens wearers	-	53%	-
Hart, <i>et al.</i> 1993	US	49 soft contact lens wearers	38%	-	-
Gray, <i>et al.</i> 1995	New Zealand	15 rigid & 86 soft contact lens wearers	-	81%	-
Rosenthal, <i>et al.</i> 1995	US	110 contact lens wearers	-	25%	-
Lipener, <i>et al.</i> 1995	Brazil	15 soft contact lens wearers	80%	87%	60% tips of bottles
Midelfart, <i>et al.</i> 1996	Norway	1 rigid & 20 soft contact lens wearers (medical students)	24%	-	-
Velasco, <i>et al.</i> 1996	Spain	126 soft contact lens wearers	84%	81%	63%
Sweeney, <i>et al.</i> 1999	Australia	40 soft contact lens wearers	-	-	26% contents 55% tips of bottles
Boost, <i>et al.</i> 2005	Hong Kong	41 ortho-k lens wearers	39%	34%	-
Cho, <i>et al.</i> 2009	Hong Kong	38 ortho-k lens wearers	29%	58%	19%
Yung, <i>et al.</i> 2007	Hong Kong	101 soft contact lens wearers	9%	34%	11%
Willcox, <i>et al.</i> 2010	Australia	232 soft contact lens wearers		84.5%	

Most organisms isolated were normal microbiota which colonize the conjunctival sac or lid skin, including CNS, *Micrococcus*, *Propionibacterium*, *Corynebacterium*, and *Bacillus* (Velasco, *et al.* 1996, Boost, *et al.* 2005, Yung, *et al.* 2007, Cho, *et al.* 2009). *S. epidermidis* was the most commonly isolated species from contact lenses (Rahim, *et al.* 2008, Szczotka-Flynn, *et al.* 2010). Some Gram negative and other pathogenic organisms were also isolated from lenses (Boost, *et al.* 2005, Szczotka-Flynn, *et al.* 2010). However, they were only occasionally identified and in a smaller percentage of subjects.

Handling contact lenses is thought to be the main source of contact lens contamination. These contaminants are most likely to be transmitted to the contact lenses through contact with hands or facial skin during contact lens handling, lens insertion, or lens removal (Szczotka-Flynn, *et al.* 2010). However, microorganisms transported from hands usually can not survive and colonize permanently on the lens surface and a normal, healthy eye. Several studies demonstrated that the level of bacterial contamination of contact lenses before insertion was quite different with the level of contamination after wear. Mowry-McKee and co-workers conducted a study to evaluate the level of contamination of contact lenses before and after wear. A significantly lower level of contamination was found on handle-and-wear lenses than on handle-only lenses (Mowrey-McKee, *et al.* 1992). This further supports the idea

that under normal conditions, there is a potent antimicrobial system in the eye which is capable of destroying most organisms left on the contact lenses during handling and insertion.

Bacterial contamination of contact lens is heavily influenced by its adhesion to the lens surfaces that is mainly affected by different material characteristics. The reason why the incidence of MK is significant higher in soft lens wearers than in RGP wearers may be partly due to lower capability of bacterial adhesion to RGP lenses. Several *in vivo* studies reported significantly lower contamination was detected on RGP lenses than soft contact lenses (Willcox, *et al.* 2001, Dutta, *et al.* 2012).

For the purposes of getting clear vision with comfortable and safe use, materials of soft contact lenses and their physical properties have been modified substantially over the past decades. The Federal Drug Administration (FDA) commonly classifies soft contact lens materials into four groups based on their charge and water content. Materials of commercially available soft contact lenses in Hong Kong are listed in Table 1.6 according to FDA Group.

Table 1.6. Materials of commercially available soft contact lenses in Hong Kong.

FDA Classification	Water Content	Charge	Available Materials
Group I	Low (<50% water)	Non-Ionic (no Charge)	Ploymacon (38% water); pHEMA (38-49% water); Lotrafilcon A (24% water); Lotrafilcon B (38% water); Galyfilcon A (47% water); Senofilcon A (38% water); Enfilcon A (46% water); Comfilcon A (48% water); Narafilcon A (46% water); HEMA (38% water)
Group II	High (>50% water)	Non-Ionic (no Charge)	Alfilcon A (66% water); Omafilcon A (62% water); Nelfilcon A (69% water); Hilafilcon A (70% water); Hilafilcon B (59% water); Hioxifilcon A (58% water)
Group III	Low (<50% water)	Ionic (Charge)	Phemfilcon A (38% water); Balafilcon A (36% water)
Group IV	High (>50% water)	Ionic (Charge)	Etafilcon A (58% water); Ocufilecon D (55% water); Phemfilcon A (55% water); Methafilcon A (55% water); Vilfilcon A (55% water); Ocufilecon B (52% water); Filcon 1b/II2/II3/IV (58% water)

In general, non-ionic polymer lenses were more favourable for the attachment of microorganisms than those composed of ionic polymer. Several studies demonstrated that non-ionic lenses had a higher level of adhesion of *P. aeruginosa* and *Staphylococcus* than ionic lenses (Henriques, *et al.* 2005, Kodjikian, *et al.* 2008, Ajayi, *et al.* 2012, Dutta, *et al.* 2012). Henriques *et al.* (2005) found that *P. aeruginosa* and *S. epidermidis* showed greater adhesion capabilities to low water content and non-ionic lenses (Lotrafilcon A) than to low water content and ionic lenses (Balafilcon A) and conventional hydrogel lenses (Galyfilcon A). Kodjikian and co-workers reported that both *S. epidermidis* and *P. aeruginosa* showed strong adhesion to the non-ionic lenses composed of Lotrafilcon B than those of Balafilcon A (Table 1.6).

Investigations of bacterial adhesion to contact lenses based on water content gave remarkably consistent results showing that bacterial adhesion increased as the water content decreased (Ahanotu, *et al.* 2001, Garcia-Saenz, *et al.* 2002, Kodjikian, *et al.* 2008, Ajayi, *et al.* 2012, Dutta, *et al.* 2012). Ajayi and co-workers reported that *P. aeruginosa* strains showed decreasing adhesive strengths to four types of contact lenses, lotrafilcon B (37.5 ± 8.2), polymacon (28.6 ± 6.3), methfilcon A (26.8 ± 5.5) and omafilcon A (23.2 ± 5.5) respectively. Garcia-Saenz *et al.* (2002), Ahanotu *et al.* (2001), and Kodjikian, *et al.* (2008) reported a similar trend for strains of

Staphylococcus.

After the introduction of silicone hydrogel lenses in 1999, several problems related with hypoxia were overcome. Some studies have been performed to differentiate contamination of contact lens between hydrogel and silicone hydrogel lenses.

Kodjikian *et al.* (2008) showed that adhesion of *P. aeruginosa* and *S. epidermidis* to three types of silicone hydrogel contact lenses (Lotrafilcon B, Balafilcon A, and Galyfilcon A) was significantly higher than that of conventional hydrogel contact lenses (Etafilcon A). Silicone hydrogel lenses showed higher levels of bacterial adhesion than conventional hydrogel lenses, which may explain why the incidence of MK is higher among silicone hydrogel lens wearers (Kodjikian, *et al.* 2008).

More recently, the number of cosmetic contact lenses users has increased greatly worldwide, especially in Asian countries including Taiwan, Korea, Singapore, and China (Rah, *et al.* 2013, Morgan, *et al.* 2015). Several recent studies have reported the relative risk of MK was greatly increased with improper use of cosmetic contact lenses (Sauer, *et al.* 2011, Singh, *et al.* 2012). In addition to non-compliant behaviors, pigments of cosmetic contact lenses are also a major factor that might increase the incidence of contact lens induced keratitis. Many cosmetic contact

lenses have their pigments on the surface which can result in significantly higher adherence of *S. aureus* and *P. aeruginosa* (Chan, *et al.* 2014).

A large variety of deposits, such as proteins, lipids, and mucin, are contained in the tears, and can accumulate on contact lenses during wear (Luensmann, *et al.* 2012, Ng, *et al.* 2013). These deposits on the lens surface may change the characteristics of contact lenses and affect bacterial adhesion to the lenses. It has been demonstrated that adhesion of *P. aeruginosa* to lenses was increased by addition of mucin, lactoferrin, lysozyme, immunoglobulin A, and bovine serum albumin, or a mixture of these compounds (Miller, *et al.* 1988). Subbaraman *et al.* (2011) found that absorption of lysozyme *in vitro* could promote the adhesion of *S. aureus* to a S contact lens (Subbaraman *et al.* (2011).

The surface properties of worn lenses were shown to differ from those of new lenses (Santos, *et al.* 2008, Willcox, *et al.* 2001), which may have a significant role in their susceptibility to bacterial adhesion. However, the influence of lens wear on bacterial adhesion remains controversial. Santos *et al.* (2008) found that balafilcon A lenses became less hydrophobic and less susceptibility to bacterial adhesion after lens wear, but conventional hydrogel (etafilcon A) lenses became more hydrophobic and also

more susceptible to bacterial adhesion ($p < 0.05$). Willcox *et al.* (2001) found that worn balafilcon A lenses contributed to a significantly increased bacterial adhesion (243% to 1393%) compared to unworn lenses, whereas wear of etafilcon A lenses resulted in a decrease in bacterial adhesion (22-48%). Borazjani *et al.* (2004) reported that the degree of *P. aeruginosa* adhesion to balafilcon A lenses worn for one week was similar to that of unworn balafilcon A lenses. These differences may be related to different lenses and lens care regimens involved, different bacterial strains, methods to assess adhesion and different compliance between individuals.

Contaminated contact lenses are thought to act as a vector transporting pathogens into the eyes (Boost, *et al.* 2005, Yung, *et al.* 2007, Szczotka-Flynn, *et al.* 2010). These results indicate that the adherence of microorganisms to the lenses can be greatly influenced by several factors, such as lens handling, material, and lens deposits (Cho, *et al.* 2009, Subbaraman, *et al.* 2011, Dutta, *et al.* 2014).

1.19.2 Contamination of lens cases

Microbial contamination of contact lens cases is commonly detected in asymptomatic wearers and is a significant risk factor for MK in contact lens wearers.

Similar to contact lens contamination, there is also a large variation in the contamination rates of lens cases. Reports of contamination rates of lens cases ranged from 25% to 87% (Table 1.5). Among contact lenses, lens cases, and lens care solutions, lens cases are usually found to be the most frequently contaminated items and are associated with a wider range of species of microorganisms (Yung, *et al.* 2007, Cho, *et al.* 2009, Szczotka-Flynn, *et al.* 2010). According to several reports, CNS was the most commonly isolated microorganism, whereas other skin microbiota, such as *Bacillus*, *Enterobacter*, *Serratia*, and *Klebsiella*, were also isolated (Boost, *et al.* 2005, Yung, *et al.* 2007). *Acanthamoeba* was also occasionally detected from lens cases. Several studies have reported the rate of *Acanthamoeba* contamination in contact lens cases, which was 4.2% in Korea, 8% in New Zealand, and 1% in Hong Kong (Gray, *et al.* 1995, Jeong, *et al.* 2005, Boost, *et al.* 2008).

Contact lens wearers are usually more aware of the hygiene of their contact lenses, and have better compliance with their care than for their lens cases. Inadequate care of contact lens case is commonly observed in up to 72% of contact lens wearers (Radford, *et al.* 1993, Yung, *et al.* 2007, Cho, *et al.* 2009). There were reports showing that many contact lens wearers did not discard disinfecting solutions and air dry their lens cases after lens disinfection, or change their lens cases regularly (Claydon, *et al.* 1994, Cho, *et al.* 2009, Wu, *et al.* 2010, Wu, *et al.* 2015). Washing the lens cases with

tap water and keeping case wet may allow for contamination of lens case by *Acanthamoeba*, as tap water is one of the sources of this organism (Jeong, *et al.* 2005, Jeong, *et al.* 2007, Boost, *et al.* 2008). It has been suggested that lens cases should be changed monthly and that users should avoid contact of the lens case with tap water to prevent lens case contamination and reduce the risk of MK (Seal, *et al.* 2003). Studies have demonstrated that contamination rates of lens cases can be reduced by good contact lens hygiene behaviors, such as air-drying facing down, tissue wiping, washing hands with soap and water, and matching the disinfecting solution with lens case (Willcox, *et al.* 2010, Wu, *et al.* 2011, Wu, *et al.* 2015).

Biofilm is a common risk factor for the contamination of lens cases (McLaughlin-Borlace, *et al.* 1998) It is well known that bacteria within the biofilm become much more resistant to lens care disinfecting solutions (May, *et al.* 1995, Szczotka-Flynn, *et al.* 2009). Biofilm formation on lens cases has been demonstrated and several lens case hygiene practices have been evaluated for the removal of biofilm formed within contact lens cases. Wu and co-workers found that biofilms within a silver-impregnated lens case were effectively removed after rubbing and rinsing with disinfecting solution, and tissue wiping. However, if only air-drying or closing the lens case lids after use were employed without any additional cleaning methods, there was little or no effect on removing the biofilm on the surface of lens cases (Wu,

et al. 2010, Wu, *et al.* 2011).

Contamination of contact lens cases is still frequently detected in contact lens wearers with good compliance (Yung, *et al.* 2007, Wu, *et al.* 2011). Recently, silver-impregnated cases have been developed as a possible solution to decrease the level of contamination of lens cases (Amos, *et al.* 2006, Dantam, *et al.* 2012). Amos, *et al.* (2006) reported that the silver lens cases had a significantly lower rate of contamination than the control (non-silver cases) (26% vs. 67%; $p \leq 0.03$). Dantam, *et al.* (2012) found that there was a significantly lower level of contamination in silver cases compared to regular cases [1.7 vs. 4.1 log colony forming units (CFU) per well; $p < 0.05$]. However, it was found that there were some variations in the antimicrobial activity of different silver-impregnated lens cases. MicroBlock case (CIBA Vision Corporation, Atlanta, USA) showed higher activity against Gram-negative bacteria, including *P. aeruginosa* and *S. marcescens*, whereas i-clean case (Sauflon Pharmaceuticals Ltd., London, UK) had more effect against *S. aureus* (Dantam, *et al.* 2011, Dantam, *et al.* 2012).

Different rates and types of lens storage case contamination were observed in contact lens wearers using different MPS (Devonshire, *et al.* 1993, Willcox, *et al.* 2010).

Willcox *et al.* (2010) found that the highest contamination rate (92%) was detected in lens cases disinfected by AQuify (0.0001% PHMB), and the lowest rate (76%) was found in cases exposed to Opti-Free Express (0.001% Polyquad and 0.0005% aldox). The colony numbers of all organisms extracted from lens cases exposed to different MPS varied, but followed the trend: Opti-Free Replen-iSH > AQuify > ClearCare > Opti-Free Express. These differences may be not simply due to the disinfectants used in MPS, as solutions may contain the same disinfectants at the same concentrations (Table 1.7). The level and frequency of lens case contamination may also be influenced by other components of the solutions. Lens cases exposed to Opti-Free Express showed a significantly decreased rate of gram negative bacteria contamination compared with Opti-Free RepleniSH (Willcox, *et al.* 2010). Boost *et al.* (2006) found that Boston Advance showed much higher antimicrobial efficacy, especially to *Serratia marcescens*, than Boston Simplicity. These differences may be due to the addition of ethylenediaminetetraacetic acid (EDTA) in the solutions (Boost. *et al.* 2006, Willcox, *et al.* 2010). EDTA is a chelating agent, also used as a weak disinfectant towards a broad-spectrum of microorganisms (Juda, *et al.* 2008, Finnegan, *et al.* 2015). Addition of EDTA to variously preserved solutions has been known to improve the killing rate to *Pseudomonas*, *Serratia* and *Staphylococcus* species (Mannis 2002, Chung, *et al.* 2003, Willcox, *et al.* 2010, Finnegan, *et al.* 2015).

1.19.3 Contamination of lens care solutions

Lens care solutions are also frequently contaminated and act as a source of organisms that may contaminate the lens cases and the lenses possibly leading to corneal infection. Contamination rates of lens care solutions reported have ranged from 11% to 63%, but are usually lower than those of contact lenses and lens cases (Table 1.5). CNS are the most frequently isolated organisms, but potential pathogens such as *S. aureus* and *P. aeruginosa* are also common contaminants of lens care solutions (Lipener, *et al.* 1995, Velasco, *et al.* 1996).

The contamination of lens care solutions commonly occurs as a result of squeezing the contents through contaminated bottle tips (Collins, *et al.* 1994, Sweeney, *et al.* 1999, Cho, *et al.* 2009). After squeezing the contents, the internal solutions are easily contaminated through suction of organisms adhering to the tip. Sweeney *et al.* (1999) suggested microorganisms may probably gain access to the bottles via contact of the nozzle with hands. The screw tops or caps of the solution bottles were more likely to be contaminated when compared with the contents. Cho *et al.* (2009) investigated contamination of solutions by using a needle syringe to collect the solution by perforating the side of the solution bottle about (2 to 5mm from the

bottom), thus avoiding cross-contamination which may be a better technique for investigating contamination of the solution (Cho, *et al.* 2009). They showed that the contamination rate of lens care solution was 19%, which was lower than previous reports (Table 1.5).

In addition, lens care solutions used by soft contact lens wearers and opened for a longer period of time are more likely to be contaminated (Panthi, *et al.* 2014). The contamination rate of solutions used for less than three months was significantly lower than those used for more than three months (10.9% versus 39.1%, $p=0.003$).

The increased contamination rates of care solutions may be also be due to significantly reduced bactericidal activity of MPS after two months of opening (Leung, *et al.* 2004). Therefore, since a bottle was opened, the length of service time may be a major risk factor for the level of contamination of care solutions.

1.20 Contact lens multipurpose solutions

Various factors have been reported as being responsible for contact lens-related MK. Microbial contamination of contact lenses and lens cases is frequently detected in contact lens wearers, which is a known risk factor for MK. To reduce the incidence

of ocular infections that occur with contact lens wear, the use of contact lens disinfecting and cleaning systems are required (Boost, *et al.* 2006, Boost, *et al.* 2010, Hildebrandt, *et al.* 2012). Currently, most (>90%) contact lens (including soft contact lens and rigid lens) users use MPS for this purpose (Lievens, *et al.* 2006, Morgan, *et al.* 2015). MPS is generally recognized as a solution with multiple active ingredients, which has several functions, including daily cleaning, rinsing and chemical disinfection of contact lenses (Bennett, *et al.* 2005).

During the past decade, MPS has been improved in cooperation with chemists, microbiologists, and contact lens material experts, making MPS more comfortable, effective, and safe (Bennett, *et al.* 2005, Epstein 2007). Manufacturers commonly produce more than one MPS for different contact lenses and there are currently almost 20 brands produced by several manufacturers.

The active ingredients of 1 MPS commonly used in Hong Kong are shown in Table 1.7. MPS comprise one or two preservatives together with buffers, surfactants, chelating, wetting, and lubrication-enhancing agents. The preservatives should achieve sufficient antimicrobial efficacy for safe use of contact lenses, while the combination of other agents may also affect the physical properties of the solution,

influencing its antimicrobial efficacy and a patient's comfort (Bennett, *et al.* 2005).

1.20.1 Active disinfecting ingredients

All MPS consist of one or two active ingredients (e.g., preservatives or antimicrobial agents) at a sufficient concentration to kill common bacterial contaminants (Boost, *et al.* 2006, Zhu, *et al.* 2007, Ward 2011). Several studies have evaluated the efficacy of these solutions and found that potential pathogens on the surface of a contact lens were significantly destroyed within the manufacturer's minimum recommended disinfection time (Boost, *et al.* 2006, Boost, *et al.* 2010). The majority of MPS utilize the preservative PHMB or quaternary ammonium compound polyquaternium alone or in combination with others disinfecting agents, such as CHX, aldox (myristamidopropyl dimethylamine), or alexidine dihydrochloride (Table 1.7).

Table 1.7 Summary of active ingredients of commonly used multipurpose solutions in Hong Kong.

MPS	Manufacturer	Main active ingredients
ReNu fresh	Bausch+Lomb	Polyaminopropyl biguanide 0.0001%
Bio-True	Bausch+Lomb	Polyaminopropyl biguanide 0.00013%/ Polyquaternium 0.0001%
Opti-Free Pure Moist	Alcon	Polyquaternium-1 0.001%/ ALDOX 0.0006%
Opti-Free RepleniSH	Alcon	Polyquaternium-1 0.001%/ ALDOX 0.0005%
Opti-Free Express	Alcon	Polyquaternium-1 0.001%/ ALDOX 0.0005%/ EDTA 0.05%
SoloCare AQUA	CIBA VISION	EDTA/ Polyhexanide 0.0001%
Revitalens	AMO	Alexidine dihydrochloride 0.00016%/ Polyquaternium-1 0.0003%
COMPLETE	AMO	EDTA/ PHMB 0.0001%
TotalCare	AMO	EDTA/ PHMB 0.0001%
Boston Advance	Bausch+Lomb	CHX 0.003%/ PHMB 0.0005%/ EDTA 0.05%
Boston Simplus	Bausch+Lomb	CHX 0.003%/ PHMB 0.0005%
MeniCare Plus	Menicon	EDTA/ PHMB 0.0005%

ALDOX: myristamidopropyl dimethylamine; EDTA: edetate disodium/ethylene diamine tetraacetic acid; PHMB: polyhexamethylene biguanide; CHX: chlorhexidine gluconate;

1.20.1.1 Polyhexamethylene biguanide

PHMB, also known as polyaminopropyl biguanide, is one of the most commonly used disinfectants (Romanowski, *et al.* 2013). It has been used in several MPS, such as Complete (Abbott Medical Optics, CA, USA) and Boston Advance (Bausch+Lomb, NY, USA) and has historically been a component of swimming pool cleaners, skin disinfectants, and perioperative cleansing products (McDonnell, *et al.* 1999, Boost, *et al.* 2006).

PHMB is a membrane-active agent that exhibits its lethal action to microorganisms by causing non-specific alterations in the integrity of the outer membrane, thereby disrupting its permeability and resulting in the complete loss of membrane function (McDonnell, *et al.* 1999). Its antimicrobial activity is unaffected by pH, sunlight, or temperature. This stability permits it to keep its activity for longer periods of time (Romanowski, *et al.* 2013). The concentration of PHMB in MPS is usually 0.0001-0.005% (1-5 µg/mL). At these concentrations, PHMB has been shown to have minimal cytotoxicity to human corneal cells (Choy, *et al.* 2013). Studies have shown that PHMB is an effective antimicrobial agent with a broad spectrum (Boost, *et al.* 2010, Yanai, *et al.* 2011). Its antimicrobial effectiveness has been also demonstrated on both the trophozoite and the cyst forms of *Acanthamoeba* (Hiti, *et al.* 2006).

1.20.1.2 Polyquad

Polyquad (polyquaternium-1), known as PQ-1, is a new generation polyquat and is a high molecular weight preservative (Carmona-Ribeiro, *et al.* 2013). It was formulated by Alcon in the mid-1980s and used as a preservative in MPS, including Opti-Free Express MultiPurpose Disinfecting Solution (Alcon, Fort Worth, TX, USA), Opti-Free Pure Moist MultiPurpose Disinfecting Solution (Alcon) (Table 1.7).

PQ-1 was shown induce K^+ leakage from several pathogenic bacteria and *C. albicans* and its targets are the cytoplasmic membrane of bacteria and the plasma membrane of fungi, which is similar to other QACs (Codling, *et al.* 2003, Carmona-Ribeiro, *et al.* 2013). It is commonly used at a concentration of 0.001% in MPS formulations at which it exhibits excellent antibacterial efficacy (Zhu, *et al.* 2007, Choy, *et al.* 2012). PQ-1 was shown to be effective against several bacteria, but reduced effects on fungi were observed (Codling, *et al.* 2003).

PQ-1 at a concentration of 0.001% was found to cause damage to human corneal epithelial cells and reduce the density of conjunctival goblet cells, thereby finally disturbing the stability of the tear film. However, it showed much less cytotoxicity to

the ocular surface than BAK (Labbe, *et al.* 2006, Liang, *et al.* 2012). In MPS however, these effects may be modified by the presence of other agents resulting in less cytotoxicity (Choy, *et al.* 2009, Choy, *et al.* 2012).

1.20.1.3 Chlorhexidine digluconate

CHX is a synthetic cationic bisguanide, which contains two biguanide groups and two symmetric 4-chlorophenyl rings (Mohammadi, *et al.* 2009). CHX has been commonly used in handwash, germicidal mouthwash, and pet shampoo. It is notable for its antimicrobial activity against a wide range of bacteria, including both Gram-positive and Gram-negative species (McBain, *et al.* 2003, Loeffler, *et al.* 2011, Petlin, *et al.* 2014).

CHX has been successfully used in rigid contact lens disinfection solutions and is usually included with other preservative systems at a concentration of 0.003% (Table 1.7). Its efficacy is mainly due to the damage to the outer cell layers, thereby altering the cells' osmotic equilibrium (el Moug, *et al.* 1985, McDonnell, *et al.* 1999, Mahendra, *et al.* 2014). With increased permeability of the cell wall, more CHX may be taken up into the cell, which subsequently damages the cytoplasmic or inner

membrane of bacteria or the plasma membrane of yeast. Although CHX has been reported to have an excellent spectrum of antimicrobial activity, it has limited effectiveness against yeast and fungi (Russell, *et al.* 1993). In addition, if it is used alone, the kill time is slow and lenses must be stored for a minimum of 10 hours. Therefore, it is often used in combination with other antimicrobial agents for greater effectiveness (Bennett, *et al.* 2005).

The cytotoxicity of CHX to the cornea has been observed in several studies (Hamill, *et al.* 1984, Choy, *et al.* 2013). Hamill, *et al.* (1984) demonstrated that CHX showed minimum cytotoxicity on the cornea. Even at concentrations of up to 2%, neither visible nor light microscopic corneal changes were found after direct topical application of CHX. However, Choy, *et al.* (2013) found that nearly 60% of cultured human corneal epithelial cells were damaged after the exposure to MPS containing 0.003% CHX. The presence of CHX may contribute to higher cytotoxicity of MPS to human corneal epithelial cells than PHMB.

1.20.1.4 Myristamidopropyl dimethylamine

Myristamidopropyl dimethylamine (MAPD) is a cationic amidoamine, also known as stearamidopropyl dimethylamine, N-[3-(dimethylamino) propyl] octadecanamide

or N, N-dimethyl-N'-tetradecanoyl-1, 3-propylenediamine. MAPD was found to be an effective antimicrobial agent against all three major classes of pathogens (bacteria, fungi and *Acanthamoeba*). Codling, *et al.* (2003) found that MAPD was effective against all organisms tested, particularly fungi. MAPD was shown to be an effective agent against nine clinical isolates of *Acanthamoeba* (Kilvington, *et al.* 2002). The minimum cysticidal concentrations of MAPD ranged from 6.25 to 25 mg/L, and it gave at least a 3-log cyst reduction after 6h at the concentration over 10 mg/L.

It is known that PQ-1 has excellent antibacterial activity and MAPD has excellent antifungal and antiprotozoal effect (Kilvington, *et al.* 2002, Codling, *et al.* 2003).

The two biocides seem to have different target organisms and may exhibit stronger antimicrobial effect together. Therefore, MAPD (Aldox) is used an additional disinfectant at 5 or 6 mg/L in Opti-Free Express Multi-Purpose Disinfecting Solution and Opti-Free Pure Moist Multi-Purpose Disinfecting Solution (Alcon), respectively.

The antimicrobial mechanism of MAPD is not clear. As a cationic amidoamine, it may exhibit similar action to that described for PHMB. The molecule of MAPD is much smaller than PHMB and CHX, which might permit it better penetration into the cornea and be more likely to irritate the eyes. Several studies have found that

MPS containing MAPD showed more cytotoxicity to human corneal epithelial cells (Choy, *et al.* 2009, Gorbet, *et al.* 2011, Choy, *et al.* 2012).

1.20.1.5 Alexidine dihydrochloride

Alexidine dihydrochloride is a biguanide compound, chemically similar to CHX with similar antimicrobial activity, used as an additional disinfectant at a concentration of 0.00016% in Revitalens MPS (Abbott Medical Optics, CA, USA).

It has broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. Alexidine dihydrochloride has significantly faster antibacterial activity compared with CHX. MPS containing alexidine dihydrochloride have been demonstrated to be safe, causing only minimum cytotoxic effects on cultured human corneal epithelial cells (Kilvington, *et al.* 2010).

1.20.2 New generation of multipurpose solutions

Since 2006, the contact lens solution industry has introduced three new-generation MPS for soft lenses. These products have been described as “dual disinfection” products because contain two significant chemical disinfectants (Table 1.7).

In 2006, Alcon introduced its dual disinfection formulations, Opti-Free Replenish and Opti-Free Express, which paired the preservative PQ-1 with Aldox. This combination of preservatives provides strong, broad-spectrum antimicrobial coverage (Zhu, *et al.* 2007). Subsequently, Alcon's Opti-Free Pure Moist was introduced which uses the same dual disinfection formulation, except that the concentration of Aldox was increased from 0.0005% to 0.0006% (Table 1.7) (Kilvington, *et al.* 2010, Ustunturk, *et al.* 2014).

Further dual disinfection combination MPS, Biotrue (Bausch+Lomb, NY, USA) and RevitaLens OcuTec (Abbott Medical Optics, CA, USA), were introduced in 2010. Biotrue combines PQ-1 with another classic contact lens preservative, PHMB. RevitaLens OcuTec combines PQ-1 with alexidine dihydrochloride (Table 1.7) (Ward 2011).

These combinations of preservatives provide strong, broad-spectrum antimicrobial coverage. Each has enhanced antimicrobial efficacy and meets the FDA criteria as a stand-alone MPS. These MPS show effective antimicrobial activity against some pathogens that are more difficult to destroy, such as fungi and *Acanthamoeba* (Boost, *et al.* 2010, Kilvington, *et al.* 2010, Ustunturk, *et al.* 2014).

1.20.3 Efficacy requirements for contact lens disinfectants

Contact lens disinfection plays a significant role in the safe use of contact lenses.

The use of MPS with effective disinfectant properties reduces the incidence of MK through the prevention or inhibition of potentially pathogenic organism growth on the lens surface and within the lens case. These disinfecting solutions contain preservatives or antimicrobial agents, which are effective against various pathogens including gram-negative and gram-positive bacteria and fungi (Boost, *et al.* 2010, Booranapong, *et al.* 2012). Before being released into the market, each solution must be assessed with respect to its antimicrobial efficacy according to defined criteria (ISO 14729 regulations). There are guidelines which describe extensive regulations governing the approval of contact lens disinfecting solutions (Food-and-Drug-Administration 1997).

The stand-alone test challenges each product with a standard inoculum (10^6 CFU/mL) of five representative microorganisms, including gram-negative bacteria (*S. marcescens* ATCC 13880 and *P. aeruginosa* ATCC 9027), gram-positive bacteria (*S. aureus* ATCC 6538), yeast (*C. albicans* ATCC 10231), and mold (*F. solani* ATCC 36031). Viability loss of each microorganism is determined under the recommended disinfection conditions.

According to the FDA Guidance Document in 1997, each solution is required to produce a 3-log reduction of viable bacterial cells and a 1-log reduction of viable fungal cells under the recommended disinfection conditions. If failing to meet this requirement, it needs to be further evaluated by the regimen test. Test solutions should be used in the methods and quantity recommended in product instructions. After the recommend disinfection period, each product is required to reach at least a combined 5-log reduction for all bacteria and 1-log reduction for any single bacteria. The number of viable yeasts and molds should remain at or below the initial concentration with an experimental error of ± 0.5 log. The product can be labeled as a contact lens disinfecting solution only after meeting the requirements of the Stand Alone Test or the regimen test.

1.20.4 Limitations of lens disinfectant solutions

To date, several studies have been carried out to evaluate the antimicrobial activity of MPS for soft contact lenses and RGP lenses (Boost, *et al.* 2006, Boost, *et al.* 2010, Kuzman, *et al.* 2013). All tested MPS showed good antimicrobial efficacy against bacteria and fungi and met the requirements of the ISO 14729 standard. However, the use of certain MPS was later linked to a large number of keratitis cases caused by *Fusarium* and *Acanthamoeba*.

In late 2005 and early 2006, the incidence of *Fusarium* keratitis was significantly increased among contact lens wearers in several countries, including Singapore, France, Hong Kong, and the United States (Khor, *et al.* 2006, Gorscak, *et al.* 2007, Rao, *et al.* 2007, Gaujoux, *et al.* 2008). The Centers for Disease Control and Prevention of USA reported that most of the patients had been using a new contact lens disinfecting solution, ReNu Multipurpose Solution, which was determined to be the cause of outbreak of *Fusarium* keratitis (CDC 2006). Subsequently, Rosenthal and co-workers reported that this solution showed less effect against *Fusarium* than several compared solutions, and that its antifungal activity decreased greatly during storage in the lens cases (Rosenthal, *et al.* 2006). Kilvington *et al.* (2010) found that ReNu with MoistureLoc lost 85-98% of activity against *F. solani* after evaporation to 2× and 4× concentration after 6 hours disinfection. The reduced efficacy may be due to the presence of Polyquaternium-10 in the formulation (Epstein, *et al.* 2007, Kilvington, *et al.* 2010).

From 2004 to 2007, an outbreak of *Acanthamoeba* keratitis was detected in many countries, including United States, Singapore, and China (Sun, *et al.* 2006, Por, *et al.* 2009, Verani, *et al.* 2009). The Centers for Disease Control and Prevention of USA

initiated an investigation into this outbreak. In multivariate analysis, the odds of having used AMO Complete Moisture Plus (Complete) solution was significantly greater in cases than controls. The MPS in question has been widely used to disinfect, rinse, clean, and store lenses. After laboratory examination, the solutions used by patients were not contaminated by *Acanthamoeba* (Verani, *et al.* 2009). Kilvington *et al.* (2008) found that Moisture Plus (Complete) was able to induce *Acanthamoeba* encystment. Propylene glycol in this solution was the key component responsible for this change (Kilvington. *et al.* 2008). After this outbreak, *Acanthamoeba spp.* were recommended to be added as a challenge organism for testing contact lens solutions in the stand-alone test, although this has not been included to date (FDA 2008, Verani, *et al.* 2009).

There are several similarities between the outbreaks of *Acanthamoeba* and *Fusarium* keratitis. Firstly, in both outbreaks patients were mostly soft contact lens wearers. Secondly, the main risk factor of each outbreak was the use of a certain disinfecting solution. Lastly, the two outbreaks were mainly due to the insufficient antimicrobial efficacy of disinfecting solutions. These outbreaks probably contributed to the introduction of the dual disinfection MPS described above.

1.20.5 Factors influencing the efficacy of multipurpose solutions

The antimicrobial efficacy of MPS may be affected by storage conditions and/or incorrect use. Newly-opened MPS for contact lenses showed effective activity against FDA recommended pathogens, but activity can be affected by storage conditions (Leung, *et al.* 2004, Boost, *et al.* 2006). Contact lens wearers are generally recommended to use their solutions for less than 3 months after opening the bottle. However, there are no guidelines on maintaining the efficacy of MPS. It was found that the antimicrobial efficacy of several disinfecting solutions was significantly decreased by the end of the 3-month trial and affected by the temperature of storage (Leung, *et al.* 2004). Contact lens wearers should be aware that the efficacy of opened solutions is closely related with time since opening and this efficacy may not be sustained for as long as 3 months.

The disinfectant capacity may also be affected by microbial factors. MPS are required to meet the requirements of the Stand Alone Test. In the Stand Alone Test, only five representative pathogens are included. However, some clinical and environmental isolates show significant resistance to biocides, which may be considerably different from FDA recommended strains (Russell 2003, Mohammadinia, *et al.* 2012). There is a need to investigate whether solutions are

effective against some non-FDA recommended strains. Several important ocular pathogens, such as *S. pneumoniae*, *H. influenzae*, *Enterococcus*, *Aspergillus*, and *Acanthamoeba* are not included in the Stand-Alone Test. Boost, *et al.* (2010) demonstrated that tested MPS showed sufficient efficacy against a wide range of bacteria which are not currently included in the FDA panel, such as MRSA, *S. pneumoniae*, *H. influenzae*, *Acinetobacter spp.*, and *Enterococcus spp.* However, reports on resistance to several disinfectants have also been reported by several authors (Sidhu, *et al.* 2002, Langsrud, *et al.* 2003, Zhang, *et al.* 2011).

There is a growing concern about the increasing incidence of disinfectant-resistant microorganisms worldwide (McDonnell, *et al.* 1999). The widespread use, and to a degree, the misuse of QACs can cause selective pressure on bacteria and is one of the main reasons for the development of resistance to QACs. Acquired resistance to QACs has been observed notably in staphylococci and some gram negative bacteria, which are pathogens causing MK. Bacteria are generally believed to have capable of acquiring genes that enable them to survive harsh environments (Zhang, *et al.* 2011, Poole 2012, Boost, *et al.* 2014). Therefore, the antimicrobial efficacy of MPS against strains showing increased resistance to QACs should be further investigated to insure the safety of contact lens wearers, especially for children.

1.21 Summary

This review has showed that disinfectants based on cationic surface-active agents are widely used in hospitals, clinics, and general environments to control infectious diseases and prevent the spread of potential pathogens, which has resulted in the increased prevalence of disinfectant resistance microorganisms, notably in staphylococci. Several QAC resistance genes that code for resistance to a broad range of disinfectants among various subtypes of staphylococci have been reported in clinical isolates, and in isolates from healthy general population subjects and the environment. Currently over 125 million people wear contact lenses worldwide. Most MPS is composed of QACs and/or biguanides, which showed a high level of antimicrobial activity against a broad spectrum of bacteria. Use of MPS to destroy microorganisms on the lenses and lens accessories is essential for safe use of contact lenses. Long term use of MPS may cause selective pressure on the ocular pathogens and lead to emergence of staphylococci harbouring QAC genes. However, there has been no report on the prevalence of staphylococci harbouring QAC genes in contact lens wearers. *Staphylococcus* harbouring QAC genes show less susceptibility to a broad of disinfectants and may survive after disinfection with MPS. It is important that disinfection is effective especially for safe use of orthokeratology which employs rigid lenses for myopia control.

CHAPTER 2

AIMS OF THE STUDY

With increased use of disinfectants in a wide range of environments, including hospitals, households, and the food industry, it is not surprising that acquired resistance to several disinfectants has been observed worldwide in some bacteria, notably in staphylococci. Currently, MPS are widely used among contact lens wearers and are necessary for safe use of contact lenses. It is reasonable to hypothesize that long term use of MPS will result in selective pressure for the development of disinfectant resistance on ocular pathogens, which may become less susceptible to antiseptics, including those used in MPS.

Currently, there is no data about the epidemiology of disinfectant resistant genes on strains isolated from contact lens wearers. In addition, MPS, which commonly include one or two disinfectants, are evaluated by demonstrating sufficient antimicrobial efficacy against only three FDA recommended strains of bacteria. Due to the limitations of the Stand Alone Test, there are no reports on the efficacy of MPS on strains harbouring disinfectant resistance genes.

The objectives of this PhD study were therefore:

1. To determine the distribution and frequency of QAC genes in *S. aureus* and CNS isolated from ortho-k lens and spectacle wearers in Hong Kong
2. To investigate whether there was an increased risk of presence of QAC genes in *S. aureus* and coagulase-negative staphylococci isolated from ortho-k lens wearers compared to that of spectacle wearers
3. To investigate the effects of long term use of MPS on the distribution of QAC genes in *S. aureus* and CNS isolated from new ortho-k lens wearers, over a 6-month period
4. To determine whether the presence of QAC genes led to an increased rate of antibiotic resistance and an increased MIC/MBC to antiseptics in *S. aureus* and CNS
5. To investigate the efficacy of four MPS for rigid gas permeable lenses against staphylococci harbouring disinfectant resistance genes
6. To determine whether the presence of QAC genes led to an increased MIC/MBC of four MPS in *S. aureus* and CNS.

CHAPTER 3

PRESENCE OF QAC GENES IN BACTERIA ISOLATED FROM ORTHOKERATOLOGY AND SPECTACLE WEARERS

3.1 Introduction

To prevent the spread of pathogens and control infection, disinfectants based on QACs, such as BAK, and biguanides, such as CHX, have been extensively used in hospitals and other health care settings (McDonnell, *et al.* 1999, Batra, *et al.* 2010). However, with the widespread use of these products, there is a growing concern about the emergence of disinfectant-resistant microorganisms, notably staphylococci, which may contribute to increasing difficulties in preventing infection (Johnson, *et al.* 2013). Antiseptic resistance in staphylococci is attributable to several genes that are mainly plasmid-borne and confer reduced susceptibility to cationic antiseptic agents including dyes (acriflavine, ethidium bromide), QACs, and biguanides by coding for efflux pumps, which reduce disinfectant concentration in the cell (Jaglic, *et al.* 2012, Costa, *et al.* 2013).

Currently, over 125 million people use contact lenses and lens accessories (Barr

2005). Contact lens wear, especially extended wear and overnight wear, has been recognized as a significant risk factor for the development of MK (Weissman, *et al.* 2002, Stapleton, *et al.* 2013). MK-inducing pathogens have been isolated not only from the cornea, but also from contact lenses and lens accessories used by patients (Yung, *et al.* 2007, Cho, *et al.* 2009, Konda, *et al.* 2014). Contact lenses are thought to be able to act as vectors, transporting pathogens into the eyes (Boost, *et al.* 2005, Yung, *et al.* 2007).

One important application of disinfectant use is to ensure safe contact lens wear (Boost, *et al.* 2006). To reduce the risk of contamination and possible subsequent infection of the eye, disinfecting solutions are essential to inactivate microorganisms on the lenses and lens accessories (Miller, *et al.* 2001, Boost, *et al.* 2006). Most MPS contain QACs and/or biguanides, which have a high level of activity against a wide range of common ocular pathogens (Boost, *et al.* 2010, Kilvington, *et al.* 2010). However, it is unknown whether long term use of these solutions will select for ocular pathogens with increased resistance to antiseptics, including those used in MPS. In this study, we compared the prevalence of disinfectant-resistance genes harbouring staphylococci colonizing the conjunctival sac, eyelashes, and eyelids of ortho-k lens wearers with those of spectacle wearers. In addition the prevalence of staphylococci harbouring these genes on the lenses and lens accessories of the respective groups was investigated.

3.2 Study design

3.2.1 Subject recruitment

Twenty three children (8–14 years) who were participating in an ortho-k project (myopic control study) at The Hong Kong Polytechnic University who had been wearing ortho-k lenses for at least one year were recruited. A control group of twenty children wearing spectacles for myopic correction were also recruited from the same study. The ocular health of each subject was assessed using slit lamp biomicroscopy at the first data collection visit. All subjects were in good general and ocular health. The control subjects had no history of wearing contact lenses.

Eligible subjects and guardians were informed verbally and in writing about the nature, benefits, and risks of the study. Before commencing the study, written informed consent was obtained. This study followed the Tenets of the Declaration of Helsinki revised in 2002 and ethics approval was obtained from the Departmental Research Committee of the School of Optometry.

After signing the consent form, each ortho-k lens wearer was given a new lens case and a new bottle of MPS. Each subject was instructed on the use and care of the lenses and lens care accessories. The subject started using their new lens cases and new MPS after this visit. Samples from subjects in the control group were collected

Sites of sample Collection

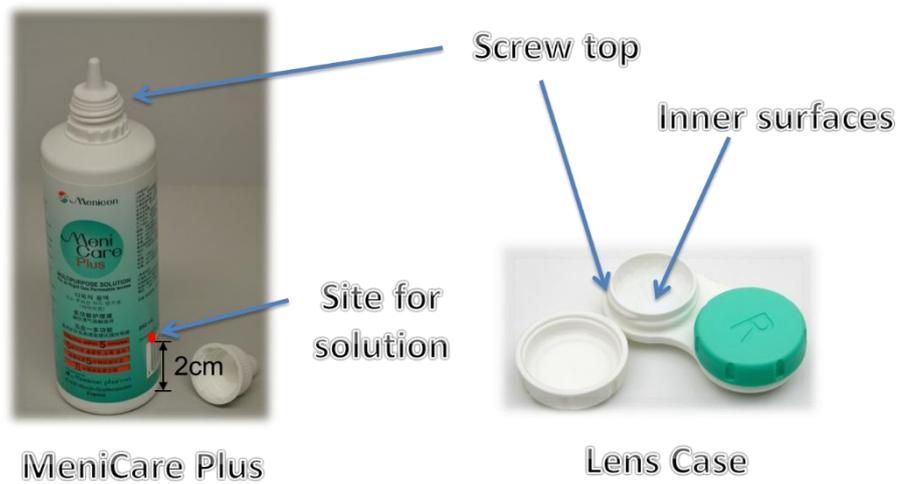


Figure 3.1 Sites of sample collection from orthokeratology lens wearers and spectacle wearers.

from the left conjunctival sac, the left eyelid and eyelashes, and the left nose pad surfaces and the root of the left arm of their spectacle frame (Figure 3.1). All samples were sent to the laboratory for microbiological culture within two hours of collection.

3.2.2 Subsequent visit (1 month later) (orthokeratology subjects only)

Two to three days before the subsequent visit, the subject was called to remind him/her to wear their lenses and clean the lens case as usual on the night before the subsequent visit. The subject brought their lenses, lens case and solutions to our Optometry clinic on the day of collection (subsequent visit). Samples were collected from the left conjunctival sac, the left eyelid and eyelashes, the lens, the lens case and lens care solutions (Figure 3.1). All samples collected were sent to the laboratory for microbiological culture within two hours of collection.

3.2.3 Methods of sample collection

3.2.3.1 Conjunctival sac

A sterile cotton swab soaked in sterile phosphate-buffered saline (PBS) was used to swab the lower conjunctiva of the left eye with a gently rolling motion from the lateral cantus toward the medial cantus. The swab was then broken off into a labeled

universal bottle containing 10mL sterile brain-heart infusion (BHI) broth (Oxoid, Basingstoke, UK).

3.2.3.2 Eyelid

A sterile cotton swab moistened in sterile PBS was used to sample the central part of the lower eyelid. The swab was broken off into a universal bottle containing 10mL sterile BHI broth.

3.2.3.3 Eyelashes

A sterile cotton swab moistened in sterile PBS was used to sample the central section of the upper eyelashes of the left eye. The swab was broken off into a universal bottle containing 10mL sterile BHI broth.

3.2.3.4 Contact lens

The contact lens worn on the left eye was removed by the subject at home as per normal routine and placed immediately into a new sterile lens case containing 2mL of sterile PBS and the case was capped and brought to the clinic for analysis.

At the clinic, the lens case containing the lens was vortexed vigorously for 30 seconds to loosen any micro-organisms adhering to the lens surface. The contact lens was then removed from the lens case with sterile forceps and returned to the subject on site. The lens extract remaining in the case was poured into a universal bottle containing 10mL of sterile BHI broth.

3.2.3.5 Contact lens case

For examination of microbiota from the routinely used lens case, a cotton swab soaked in Dey-Engley neutralizing broth (Oxoid) with 2mM EDTA was used to sample the inner surfaces and the screw tops of the lens case. The swab was broken off into a universal bottle containing 10mL sterile BHI broth. The lens case was discarded after sampling.

3.2.3.6 MPS

A sterile cotton swab moistened in sterile Dey-Engley neutralizing broth with 2mM EDTA was used to sample the screw top of the MPS bottle for microbiological culture. The swab was broken off into a universal bottle containing 10mL sterile BHI broth.

For each solution, firstly a cotton swab soaked with 75% alcohol was used to wipe the surface of the solution bottle for avoiding contamination of sample. Then a 2-mL aliquot of MPS was withdrawn from the subject's solution bottle (2cm from the bottom) using a needle syringe and transferred directly into a labeled universal bottle containing 8mL of Dey-Engley neutralizing broth with EDTA.

3.2.3.7 Spectacle frame

A sterile cotton swabs moistened in sterile PBS were used to sample the right nose pad surface and the root of the right arm of the spectacle frame. The swab was broken off into a universal bottle containing 10mL sterile BHI broth.

Finally, the bottles were vortexed for 30 seconds to release any micro-organisms adhering to the swabs.

3.2.4 Microbial assessment

All bottles of BHI broth were incubated at 37 °C for 16 hours before being subcultured on *SASelect* agar (Bio-Rad, Redmond, WA, USA) and incubated for 24h. This medium contains chromogenic substrates that help to differentiate *S. aureus* from other staphylococci. For cultures showing growth, colonies displaying

typical staphylococcal morphology were isolated, Gram-stained and identified using catalase test, tube coagulase test, and Staphaurex Plus test (Murex Biotech Ltd, Dartford, UK) for microbiological identification.

For the conjunctival sacs, both *S. aureus* and CNS were characterized as both organisms may be a cause of infection at this site. For all other samples, only the more pathogenic *S. aureus* was investigated. After identification, all bacteria belonging to *Staphylococcus* species were stored in Tryptone Soy Broth (Oxoid) containing 20% glycerol at -80 °C for further study

3.2.5 Extraction of plasmid DNA

Each strain was subcultured twice before use on nutrient agar. Plasmid DNA was extracted by a modified alkaline lysis method, with addition of lyostaphin and lysozyme (Sigma-Aldrich, St Louis, MO, USA) (Zhang, *et al.* 2011). In brief, two colonies of each strain were inoculated into an Eppendorf tube containing 200 µL lysis solution which included 10 µL lysostaphin (500U/mL) (Sigma-Aldrich), 10 µL lysozyme (5000U/mL) (Sigma-Aldrich), 4 µL EDTA (0.5M), 2 µL Tris-Hcl (1M), and 174 µL MiliQ water. The Eppendorf tubes were vortexed to homogenize the bacterial suspension and then incubated with agitation at 37 °C for 1 hour. At the end of this period, 0.5mL 1M Tris, 0.5mL 0.1M EDTA, 5mL 1M NaOH, 2.5mL 10% SDS and 41.5mL distilled water were added into a sterile bottle to prepare TENS solution. A

300 μ L aliquot of TENS was added into each Eppendorf tube, and gently mixed (up and down for 8-10 times), followed by addition of 150 μ L potassium acetate (3M) into each tube and mixed gently (up and down for 8-10 times). The tube was placed on ice for 20 minutes and then centrifuged at 11, 200g for 20minutes. The supernatant was transferred to a new Eppendorf tube to which was added 1mL 100% ethanol (cold from fridge).

The tubes were mixed gently and placed on ice for 30 minutes, followed by centrifuging at 11, 200g for 30 minutes. The supernatant in each tube was discarded. To each tube, 1mL 70% ethanol (room temperature) was added and mixed gently and centrifuged at 11, 200g for 5 minutes. The supernatant in each tube was discarded and the precipitate was allowed to air dry at room temperature for at least 10 minutes. A 50 μ L aliquot of MiliQ water was added to each tube and the contents containing the bacterial DNA was stored at -20 $^{\circ}$ C before being used in the PCR.

3.2.6 Polymerase chain reaction

PCR was carried out for detection of *smr*, *qacA/B*, G, H and J using primer sequences shown in Table 3.1 (Bjorland, *et al.* 2005, Noguchi, *et al.* 2006, Correa, *et al.* 2008, Smith, *et al.* 2008, Zhang, *et al.* 2011).

Table 3.1. Primers used for detection of QAC genes by polymerase chain reaction.

Primer	Sequence (5' →3')	Amplicon size	Reference
<i>qacA/B</i>	For (GCAGAAAGTGCAGAGTTCG)	361bp	Noguchi, <i>et al.</i> 2005.
	Rev (CCAGTCCAATCATGCCTG)		
<i>smr</i>	For (GCCATAAGTACTGAAGTTATTGGA)	195bp	Zhang, <i>et al.</i> 2011.
	Rev (GACTACGGTTGTTAAGACTAAACCT)		
<i>qacG</i>	For (CAACAGAAATAATCGGAACT)	275bp	Bjorland, <i>et al.</i> 2005.
	Rev (TACATTTAAGAGCACTACA)		
<i>qacH</i>	For (CAATAGTCAGTGAAGTAATAGGCAGTG)	295bp	Zhang, <i>et al.</i> 2011.
	Rev (TGTGATGATCCGAATGTGTTT)		
<i>qacJ</i>	For (CTTATATTTAGTAATAGCG)	306bp	Bjorland, <i>et al.</i> 2005.
	Rev (GATCCAAAAACGTTAAGA)		

The volume of each PCR reaction mixture for *qacA/B* and *smr* was 25 μL , consisting of 5 μL of 5x reaction buffer (Promega, Madison, WI, USA), 1.5mM MgCl_2 (Promega), 0.2mM dNTPs (Promega), 0.1 μM forward primer (Invitrogen, Carlsbad, CA, USA), 0.1 μM reverse primer (Invitrogen), 1unit Taq DNA polymerase (Promega), 100ng of extracted plasmid DNA, and 13.3 μL MiliQ water (Table 3.2).

Table 3.2 Mixture for amplification of *qacA/B* or *smr*.

Reagents	Concentration	Volume(μL)
Buffer	5x	5 μL
MgCl_2	25mM	1.5 μL
dNTPs	2mM	2.5 μL
Forward primer	10 μM	0.25 μL
Reverse primer	10 μM	0.25 μL
Taq DNA polymerase	5u/ μL	0.2 μL
DNA template	50ng/ μL	2 μL
MiliQ water		13.3 μL
Total		25 μL

MgCl_2 : Magnesium chloride; dNTPs: deoxyribonucleotide

Amplification of *qacA/B* and *smr* was performed using the following PCR conditions: initial denaturation at 95 $^\circ\text{C}$ for 5min, followed by 30 cycles of denaturation at 95 $^\circ\text{C}$, 30s of annealing at 53 $^\circ\text{C}$, and 60s of extension at 72 $^\circ\text{C}$.

Amplification was completed with an extension step of 72 $^\circ\text{C}$ for 5min (Table 3.3).

Table 3.3 Polymerase chain reaction conditions for amplification of *qacA/B* or *smr*.

Temperature (°C)	Time	No. of Cycles
95	5min	1
95	30s	30
53	30s	
72	1min	
72	5min	1

The PCR products were subjected to electrophoresis using a 2% agarose gel and visualized in a UV transilluminator. *S. aureus* TS77 (with *qacA/B* genes) and *S. aureus* L20 (with *smr* genes), which were kindly donated by Professor K. Hiramatsu, Juntendo University Tokyo, Japan, were used as control strains. A negative control, 2 μ L of sterile water, was included instead of template DNA.

Each reaction tube for *qacG*, *qacH* and *qacJ* contained 5 μ L (5x) reaction buffer (Promega), 1.5mM MgCl₂ (Promega), 0.2mM dNTPs (Promega), 0.2uM forward primer, 0.2uM reverse primer, 1unit Taq DNA polymerase (Promega), 100ng of extracted plasmid DNA, and 12.8 μ L MiliQ water (Table 3.4).

Table 3.4 Mixture for amplification of *qacG*, H or J.

Reagents	Concentration	Volume
Buffer	5x	5 μ L
MgCl ₂	25mM	1.5 μ L
dNTPs	2mM	2.5 μ L
Forward primer	10uM	0.5 μ L
Reverse primer	10uM	0.5 μ L
Taq DNA polymerase	5u/ μ L	0.2 μ L
DNA template	50ng/ μ L	2 μ L
MiliQ water.		12.8 μ L
Total		25 μ L

MgCl₂: Magnesium chloride; dNTPs: deoxyribonucleotide

Amplification of *qacG*, H and J were performed using the following PCR conditions: initial denaturation at 95 °C for 5min, followed by 30 cycles of denaturation at 95 °C, 30s of annealing at 56 °C, and 60s of extension at 72 °C. Amplification was completed with an extension step of 72 °C for 5min (Table 3.5). The PCR products were visualized using a UV transilluminator following electrophoresis in 2% agarose gels. *S. aureus* RN4220, which was kindly donated by Dr. J Bjorland, Oslo, Norway, was used as positive control strain for *qacG*, H, and J. A negative control, containing 2 μ L of sterile water instead of template DNA, was included.

Table 3.5 Polymerase chain reaction conditions for amplification of *qacG*, H or J.

Temperature (°C)	Time	No. of Cycles
95	5min	1
95	30s	30
56	30s	
72	1min	
72	5min	1

3.2.7 Statistical analysis

Statistical analyses were performed using SPSS system for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). Association of categorical variables was determined by Chi squared or Fisher's exact test. *p*-value of 0.05 or below was considered as statistically significant.

3.3 Results

3.3.1 Age and sex distribution

The ages of ortho-k lens wearers ranged from 8 to 14 with a mean of 11.7 years. The age range for spectacle wearers was 7 to 14 with a mean of 10.6. Of the 23 ortho-k lens wearers, 52.2% (12) were female and 47.8 % (11) were male. 55% (11)

spectacle wearers were female and 45% (9) were male. (Table 3.6) There was no significant differences in age ($p = 0.1$) or gender ($p = 0.15$) between subjects of the two groups.

Table 3.6 Age and sex distribution of orthokeratology lens and spectacle wearers.

	Ortho-k lens wearers (n=23)	Control subjects (n=20)	<i>p</i> value
Age [median (range)]	12 (8-14)	11 (7-14)	0.1*
Male [No. (%)]	11 (47.8)	9 (45)	0.85#
Female [No. (%)]	12 (52.2)	11 (55)	0.85#

*Probability values for differences between groups using Mann-Whitney tests.

#Probability values for differences between groups using Chi Square test.

3.3.2 Colonization with *S. aureus* and coagulase negative staphylococci in orthokeratology lens and spectacle wearers

A total of 159 staphylococci (110 *S. aureus* and 49 CNS) were isolated from ortho-k lens and spectacle wearers (Table 3.7). Some subjects yielded more than one strain of *S. aureus* or CNS.

Table 3.7. Origin of staphylococcal isolates.

Origin of isolates	<i>S. aureus</i>	CNS
<i>Orthokeratology group</i>		
Conjunctival sacs	5	31
Eyelid	26	
Eyelashes	12	
Contact lens	6	
Lens case	13	
MPS (screw top)	0	
MPS (solution)	0	
Total	62	31
<i>Control group</i>		
Conjunctival sacs	19	18
Eyelid	5	
Eyelashes	14	
Spectacle frames	48	
Total		18
All	110	49

Isolates of CNS only characterized from conjunctival sacs

Colonization in ortho-k lens wearers (Table 3.8): A total of 93 staphylococci (62 *S. aureus* and 31 CNS) were isolated from ortho-k subjects. *S. aureus* was present in 5 (21.7%) conjunctival sacs, 12 (52.2%) eyelids, and 8 (34.8%) eyelashes.

Contamination was present on 5 (21.7%) of contact lenses, and 6 (26.1%) of lens cases. *S. aureus* was not isolated from either the screw tops of MPS bottles or MPS. CNS was present in 19 (82.6%) conjunctival sacs.

Table 3.8 Comparison of carriage of *S. aureus* and coagulase negative staphylococci between orthokeratology lens wearers and the control group.

Organism	Position	Group (No. of subjects)	Positive No. (%)	<i>P</i>	OR	95% CI
<i>S. aureus</i>	Conjunctival sac	Ortho-k (23)	5 (21.7)	NS	0.41	0.63-9.12
		Control (20)	8 (40)			
	Eyelid	Ortho-k (23)	12 (52.2)	NS	2.03	0.14-1.69
		Control (20)	7 (35)			
	Eyelashes	Ortho-k (23)	8 (34.8)	NS	1.6	0.17-2.36
		Control (20)	5 (25)			
	Spectacle frames	Control (20)	7 (35)	NA		
	Contact lens	Ortho-k (23)	5 (21.7)	NA		
	Lens case	Ortho-k (23)	6 (26.1)	NA		
	CNS	Conjunctival sac	Ortho-k (23)	19 (82.6)	NS	2.56
Control (20)			13 (65)			

Isolates of CNS only characterized from conjunctival sacs; OR: Odds Ratio; CI: Confidence Intervals; NA: not applicable; NS: non-significant difference

Colonization in spectacle wearers (Table 3.8): A total of 66 staphylococci (48 *S. aureus* and 18 CNS) were isolated from spectacle wearers. *S. aureus* was present in 8 (40%) conjunctival sacs, 7 (35%) eyelids, and 5 (25%) eyelashes. The organism was isolated from 7 (35%) spectacle frames. CNS was present in 13 (65%) conjunctival sacs.

S. aureus isolation from the conjunctival sac was more common in spectacle wearers (40%) than in ortho-k wearers (21.7%), but *S. aureus* carriage rates on the eyelids (30% and 25%) and eyelashes (25%) of the control subjects were lower than those of ortho-k subjects. However, these values did not reach statistical significance.

The conjunctival sacs of 19 (82.6%) ortho-k subjects were colonized with CNS. In comparison, colonization was only observed in 13 (65%) of control subjects, but this difference in isolation of CNS did not reach significance.

3.3.3 Prevalence of QAC genes in *S. aureus* and coagulase negative staphylococci isolates from orthokeratology lens and spectacle wearers

QAC genes were more commonly detected in CNS (*qacA/B* 26.5%, *smr* 12.2%, and *qacH* 12.2%) than *S. aureus* (*qacA/B* 10.9%, *smr* 4.5%, and *qacH* 0.9%). No sample was positive for *qacG* or *qacJ*. However, the difference only reached statistical

significance for *qacA/B* (Odds Ratio = 0.34; Confidence Intervals: 0.14-0.81; $p = 0.015$) and *qacH* (Odds Ratio = 0.07; Confidence Intervals: 0.01-0.56; $p = 0.01$).

The number of *S. aureus* isolates carrying QAC genes was low, but these genes were more common in isolates from ortho-k subjects. Samples from lenses and lens cases yielded six and 13 *S. aureus* isolates, respectively, of which three carried QAC genes: two from the cases carried *qacA/B* and one isolate from the lenses also carried both *qacA/B* and *smr*. Spectacle frames yielded 14 *S. aureus* isolates, but none of these carried QAC genes. Carriage rates of *qacA/B* in *S. aureus* differed significantly between ortho-k lenses wearers and the control group (Odds Ratio = 4.42; Confidence Intervals: 0.92-21.24; $p = 0.046$). However, the difference in prevalence of *smr* in *S. aureus* (ortho-k 4.8%, control 4.2%) isolates from the two groups did not reach statistical significance ($p = 0.867$). Only one *S. aureus* in the ortho-k group was positive for *qacH*. (Table 3.9)

QAC genes were more frequently present in CNS from ortho-k than control subjects, but the difference only reached statistical significance for *qacA/B* (Odds Ratio = 10.74; Confidence Intervals: 1.26-91.47; $p = 0.011$). The difference in prevalence of *smr* (ortho-k 12.9%, control 11.1%) and *qacH* (ortho-k 12.9%, control 11.1%) in CNS isolates from the two groups did not reach statistical significance ($p = 0.854$). (Table 3.9)

Table 3.9 Presence of *qacA/B*, *smr* and *qacH* in isolates of *S. aureus* and coagulase negative staphylococci from orthokeratology lens wearers and the control group.

Organism	Group (No. of isolates)	<i>qacA/B</i>				<i>smr</i>				<i>qacH</i>			
		Positive No. (%)	<i>P</i>	OR	95% CI	Positive No. (%)	<i>P</i>	OR	95% CI	Positive No. (%)	<i>P</i>	OR	95% CI
CNS	Ortho-k (31)	12 (38.7)				4 (12.9)				4 (12.9)			
	Control (18)	1 (5.6)	0.011	10.74	1.26-91.47	2 (11.1)	0.854	1.19	0.20-7.22	2 (11.1)	0.854	1.19	0.20-7.22
<i>S. aureus</i>	Ortho-k (62)	10 (16.1)				3 (4.8)				1 (1.6)			
	Control (48)	2 (4.2)	0.046	4.42	0.92-21.24	2 (4.2)	0.867	1.17	0.19-7.29	0 (0)	N/A		

***S. aureus* vs CNS**

Presence of *qacA/B* OR 0.34; 95% CI 0.14-0.81 (***p* = 0.015**)

Presence of *smr* OR 0.34; 95% CI 0.1-1.18 (*p* = 0.09)

Presence of *qacH* OR 0.07; 95% CI 0.01-0.56 (***p* = 0.01**)

Isolates of CNS only characterized from conjunctival sacs; OR: Odds Ratio; CI: Confidence Intervals; *P* value in bold indicates significance; N/A: not applicable.

3.4 Discussion

The presence of QAC genes in staphylococci has previously been reported in a number of studies performed in Hong Kong (Zhang, *et al.* 2011), Japan (Noguchi, *et al.* 1999, Alam, *et al.* 2003), Europe (Mayer, *et al.* 2001), and North America (Longtin, *et al.* 2011) (See Section 1.14). This is the first study to investigate their presence in the normal microbiota of the eye and contact lenses and accessories. This study focused on the epidemiology of QAC genes in *S. aureus* and CNS isolated from ortho-k and spectacle wearers.

In this study, children aged from 7 to 14, who had been wearing ortho-k lenses for at least one year, were recruited. If there is good adherence to correct handling procedures, ortho-k lens wear can be safe to the wearers (Cho, *et al.* 2009). However, the use of ortho-k lens and MPS will influence the microbiota and increase the carriage rate of strains carrying disinfectant resistance genes, which may bring potential risks to the wearers, especially to the children.

3.4.1 Carriage of *S. aureus* in orthokeratology lens and spectacle wearers

Carriage of *S. aureus* in the periorbital region (including conjunctival sac, eyelids, and eyelashes) and accessories of ortho-k lens and spectacle wearers may act as a potential pathogen for ocular infection or play an important role in transmission of

infection. Carriage rates of *S. aureus* in children in the periorbital region, especially in the conjunctival sac (ortho-k 22% and control 40%), were higher than previously reported (range 2.2% to 16.4%) (Singer, *et al.* 1988, Fleiszig, *et al.* 1992, Hautala, *et al.* 2008). This difference may be associated with differences in nasal colonization rates of *S. aureus* (Wertheim, *et al.* 2005, Ho, *et al.* 2015). However, nasal colonization of *S. aureus* in Hong Kong (Zhang, *et al.* 2011, Ho, *et al.* 2015) are similar to or lower than those reported in other developed countries (Wertheim, *et al.* 2005, Kuehnert, *et al.* 2006). In contrast to other studies that utilized direct subculture onto the agar plates, the collected swabs in the current study were enriched in BHI broth before culture, which would allow low numbers of organisms a greater chance to be detected.

The *S. aureus* carriage rate was lower in the conjunctival sacs of ortho-k subjects than those of control subjects which may be related to the use of MPS that could kill or limit the growth of microbiota in the conjunctival sac. In contrast, carriage rates of *S. aureus* were higher on the eyelids (52%) and eyelashes (35%) of ortho-k subjects than those of spectacle controls (35% and 25%, respectively). Organisms may have been transferred from the fingers to the skin of eyelids and eyelashes during ortho-k lens insertion and removal.

S. aureus was present on 22% lenses and 26% lens cases of ortho-k subjects, which

was higher the result of previous studies (Yung, *et al.* 2007, Cho, *et al.* 2009) (See Section 1.19). Contamination of the lens was a combination of organisms from the eye and organisms adhering to the lens overnight as well as the inevitable contamination from the skin (finger) during lens removal by the subject. Subjects would usually remove their left lens after the right lens and would be unlikely to wash their hands in-between these procedures. Removal of lenses involved touching the periorbital tissues with the fingers before handling the left lens.

On the night before the visiting day, lens case was required to be cleaned and air dried as normal. The lens case was capped on the morning of the visiting day. Organisms in the lens case are likely to come from the fingers during lens removal and from the environment during air drying. In addition, the lens case was not disinfected as would be usual, permitting more organisms to survive. For examination of contamination of used lens cases, a cotton swab soaked in Dey-Engley neutralizing broth with 2mM EDTA was used to sample the inner surfaces and the screw tops of the lens case. EDTA was used to help release bacteria from biofilms present on the lens case surface, which may have increased the bacterial detection rate. At this low concentration, EDTA is not bactericidal and has been used in previous studies of contamination of lens cases (Root, *et al.* 1988, Juda, *et al.* 2008).

The nose pad and arm of the spectacle frame were directly in contact with the skin. The carriage rates of *S. aureus* on the surface of nose pad and leg of the spectacle frame (35%) were similar to that of eyelids (35%) and eyelashes (25%) of control subjects, which may reflect the skin microbiota of general population. In addition, isolation of *S. aureus* from items not directly in contact with the eye, that is, the lens cases and frames, were similar. This may represent contamination from fingers which have been in contact with nasal pharyngeal secretions, the anterior nares being the primary site of colonization for *S. aureus* (See Section 1.3)

3.4.2 Carriage of coagulase negative staphylococci in orthokeratology lens and spectacle wearers

Formerly, CNS was thought to rarely cause disease and clinicians frequently regarded isolates as contaminants. However, there is an increasing trend of CNS being associated with ocular infections (Green, *et al.* 2008, Rogers, *et al.* 2009) (See Section 1.6). In the current study, for the conjunctival sacs, both *S. aureus* and CNS were characterized, as both may be a cause of infection at this site. For all other samples, only *S. aureus* which is a more pathogenic organism was investigated.

CNS is the dominant organism in both conjunctival sacs and skin. Ortho-k lens can act as a vector transferring CNS from the skin of fingers into the conjunctival sac. Although MPS left on the lens may kill or inhibit the growth of organisms in the

conjunctival sac, quite a number of CNS survived in the conjunctival sac. In contrast with *S. aureus*, the carriage rate of CNS was higher in conjunctival sac of ortho-k subjects (82.6%) than that of control subjects (65%). But there was no significant difference in the carriage rate of CNS between the two groups.

3.4.3 Prevalence of QAC genes in *S. aureus* isolates

Several *in vitro* studies have demonstrated that the expression of QAC genes can be induced by the selective pressure from use of sub-lethal concentrations of disinfectants and these genes can spread between *Staphylococcus* spp (Smith, *et al.* 2008, Zhang, *et al.* 2011) (See Section 1.14).

In this study, we found that QAC genes were detected in isolates from both ortho-k lens and spectacle wearers, but the rate of *S. aureus* isolates carrying QAC genes was higher in ortho-k subjects than that of control subjects. In the ortho-k group, 11 *S. aureus* isolates harbouring QAC genes were found, including 8 *qacA/B*, 1 *smr*, 1 *qacA/B+smr*, and 1 *qacA/B+smr+qacH*. In contrast, only 2 *S. aureus* were isolated with *smr* or *qacA/B+smr* from the control group.

There were five and 10 *S. aureus* isolates from the conjunctival sacs of ortho-k and control subjects, respectively, of which only one from ortho-k group carried *qacA/B*.

Although the carriage rate of *S. aureus* was lower in ortho-k group than the control group, prevalence of QAC gene positive strains was found to be higher in ortho-k subjects, which may have resulted from the selective pressure of the use of MPS.

Similar results were found in *S. aureus* isolated from eyelids and eyelashes between ortho-k and control group. Ortho-k subjects yielded three QAC positive isolates whereas only one gene positive isolate was retrieved from the control group. In addition, one isolate from the lenses harboured *qacA/B+smr* and two of the isolates from lens cases carried *qacA/B*, whilst none of those from spectacle frames were positive for antiseptic resistance genes. In this study, isolates were collected from the lens and inner surface and screw top of the lens cases and nose pad and root of the arm of spectacles, which were frequently touched by the skin of hands. These isolates can partly reflect the microbiota of the skin of each subject. Compared to control subjects, the hands of ortho-k lens wearers will inevitably come into contact with MPS, which will frequently be diluted by tap water or saline. This will bring selective pressure to organisms on the skin over the long term and increase the opportunities to acquire QAC genes, which helps to explain the higher carriage rate of QAC genes in ortho-k lens wearers.

Carriage rates of *qacA/B* in *S. aureus* differed significantly between ortho-k lenses wearers and the control group (Odds Ratio = 4.42; Confidence Intervals: 0.92-21.24;

$p = 0.046$). However, the difference in prevalence of *smr* in *S. aureus* (ortho-k 4.8%, control 4.2%) isolates from the two groups did not reach significance ($p = 0.867$). This, once again, suggests selection of strains by disinfection exposure in the ortho-k subjects, although the source of the *S. aureus* contamination could not be verified as nasal sampling was not performed.

QacG, *qacH* and *qacJ* are less commonly present in *S. aureus* isolates from humans which is similar to the results of the current study (Zhang, *et al.* 2011, Ye, *et al.* 2012) (See Section 1.14). Only one *S. aureus* carrying *qacH* was found in the ortho-k group and no *S. aureus* harbouring *qacG* or *qacJ* was detected. A larger sample size may result in more isolations of these genes, but their occurrence in isolates from the general population in Hong Kong appears to be low (Zhang *et al.*, 2011).

3.4.4 Prevalence of QAC genes in coagulase negative staphylococci isolates

The prevalence of QAC genes in *S. aureus* has been well documented in previous studies (Zhang, *et al.* 2011). Most disinfectant resistance genes are plasmid-borne, and can spread between staphylococcal species. CNS, the most common organism in normal microbiota, may have a higher potential to harbour these genes, as several studies performed in recent years have reported that QAC genes were more commonly detected in CNS than *S. aureus* (Sidhu, *et al.* 2002, Zhang, *et al.* 2011, Zhang, *et al.* 2012). The increased prevalence of disinfectant resistance genes may

play a significant role in the increasing incidence of infectious diseases associated with CNS.

In this study, CNS carrying QAC genes were isolated from 16 ortho-k lens wearers and four control subjects. The prevalence of QAC genes in CNS was higher than that of *S. aureus*. But the difference only reached statistical significance for *qacA/B* (Odds Ratio =0.34; Confidence Intervals: 0.14-0.81; $p = 0.015$). There were no strains harbouring *qacG* or *qacJ*. In further studies should examine the carriage of CNS from the periocular region in more detail to investigate relationships between CNS and QAC genes.

In summary, long term use of antiseptics may contribute to a higher presence of QAC genes among staphylococci isolated from ortho-k lens wearers. This may increase the risk of infection with antiseptic resistant organisms. It is important that disinfection of lenses is performed correctly, avoiding dilution of disinfection solution by topping up or use of expired solutions which may have reduced disinfecting power enhancing the survival of organisms. However, MPS will still frequently be diluted by tap water or saline, and be left on the skin of hands and eyelids, which will bring selective pressure to the colonies and lead to a higher risk of spread of biocide resistance genes. Therefore, disinfecting solution with less selective pressures to the organism, such as hydrogen peroxide system, may be

preferable and this needs to be further investigated.

Paper published

Shi GS, Boost MV, Cho P. Prevalence of antiseptic-resistance genes in staphylococci isolated from orthokeratology lens and spectacle wearers in Hong Kong. *Investigative Ophthalmology and Visual Science*. 2015; 56(5): 3069-3074.

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CHAPTER 4

PRESENCE OF QAC GENES IN BACTERIA ISOLATED FROM ORTHOKERATOLOGY LENS WEARERS OVER A SIX-MONTH PERIOD

4.1 Introduction

Cationic antibacterial disinfectants have been extensively used in hospitals and other health care settings for a variety of clinical purposes and play an important role in the control of infectious diseases due to their wide range of effectiveness and low toxicity (Gilbert, *et al.* 2005, Grare, *et al.* 2010). Their widespread use, and to a degree, the misuse of these agents can cause selective pressure on bacteria and is one of the main reasons for the development of resistance to disinfectants (Sidhu, *et al.* 2002).

Bacteria have always been capable of acquiring genes that enable them to survive harsh environments. Acquired resistance to QACs has been observed worldwide, notably in staphylococci (Longtin, *et al.* 2011, Zhang, *et al.* 2011, Zhang, *et al.* 2012) (See Section 1.14).

In recent years, an increase in ocular infection related to contact lens wear has been observed. Staphylococci, the most common pathogens associated with ocular

infections, are commonly isolated not only from the ocular surface of asymptomatic lens wearers, but also from contact lens and lens accessories (Boost, *et al.* 2005, Cho, *et al.* 2009, Konda, *et al.* 2014) (See Section 1.19).

To reduce the risk of contamination (and subsequent infection of the eye), disinfecting solutions are used to inactivate microorganisms on the lenses and lens accessories (Boost, *et al.* 2010). In chapter 3, it was reported that several disinfectant resistance genes, such as *qacA/B*, *smr* and *qacH*, were widely distributed among staphylococcal isolates from ortho-k lens wearers and control subjects. The higher incidence of *qacA/B* detected among the staphylococci isolated from ortho-k subjects may be due to selection pressure from long term use of disinfectants containing cationic antiseptic agents. However, the ortho-k subjects recruited had been wearing ortho-k lenses for at least one year. There appears to be no published data on the process of the increase in frequency of disinfectant-resistance genes under the selective pressure of long term use of MPS.

In this study we observed the frequency of disinfectant-resistance genes in staphylococci isolated from the conjunctival sacs, eyelids, eyelashes, lenses, and lens accessories of ortho-k lens wearers over a 6-month period.

4.2 Materials and methods

4.2.1 Subject recruitment

Seventeen children (7–14 years) who were participating in an ortho-k project (myopic control study) at The Hong Kong Polytechnic University were recruited. The ocular health of each subject was assessed using slit lamp biomicroscopy at the first data collection visit. All subjects were in good general and ocular health and had no prior contact lens wear history.

Eligible subjects and guardians were informed verbally and in writing about the nature, benefits and risks of the study. Written informed consent was obtained before commencing the study. This study followed the Tenets of the Declaration of Helsinki revised in 2002 and ethics approval was obtained from the Departmental Research Committee of the School of Optometry.

4.2.2 Study design

4.2.2.1 Visit 1

After signing the consent form, samples from each subject were collected from the left conjunctival sac, the left eyelid and eyelashes, and the left nose pad surfaces and

the root of the left arm of the spectacle frame (Figure 3.1) and sent to the laboratory for microbiological culture within two hours of collection.

4.2.2.2 Subsequent visits

After commencing ortho-k lens wear, sample collection was conducted after three months and six months of lens wear. Each subject was instructed on the use and care of the lens and lens care accessories. The subject was required to replace the lens case and solutions every month.

For these subsequent visits, subjects were called two to three days before the subsequent visit to remind them to wear the lenses and clean the lens case as usual on the night before attending the clinic. Subjects were required to bring their lens case, solution, and lenses to the Optometry clinic on the data collection day. Samples were collected from the left conjunctival sac, left eyelid and eyelashes, lens, lens case, and lens care solution (Figure 3.1). All samples collected were sent to the laboratory for microbiological culture within two hours of collection.

The procedures of sample collection and microbial assessment, and detection of *qac* genes (DNA extraction and PCR) were performed as described in Chapter 3.

4.2.3 Statistical analysis

Statistical analyses were performed using SPSS system for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). Association of categorical variables was determined by Chi squared or Fisher's exact test. *p*-value of 0.05 or below was considered as statistically significant.

4.3 Results

4.3.1 Overview of the subjects

The age of ortho-k lens wearers ranged from 7 to 14 with a mean of 10.9 years. Of the 17 ortho-k lens wearers, 52.9% (9) were female and 47.1 % (8) were male. (Table 4.1)

In this study, two subjects dropped out because of no time for aftercare after the 3-month visit and 15 subjects completed the study.

Table 4.1 Age and sex distribution of orthokeratology lens wearers.

Subjects	No (%)	Age (years)	
		Range	Mean \pm SD
Male	8 (47.1)	10-14	11.9 \pm 0.8
Female	9 (52.9)	7-14	10.0 \pm 2.4
Total	17 (100)	7-14	10.9 \pm 2.0

4.3.2 Colonization with *S. aureus* and coagulase negative staphylococci in orthokeratology lens wearers over time

A total of 183 staphylococci (116 *S. aureus* and 67 CNS) were isolated from ortho-k wearers (Table 4.2).

Table 4.2 Origin of staphylococcal isolates.

Origin of isolates	<i>S. aureus</i>	CNS
Baseline		
Conjunctival sacs	9	15
Eyelid	18	
Eyelashes	5	
Spectacle frames	13	
Total	45	15
3-month		
Conjunctival sacs	3	28
Eyelid	23	
Eyelashes	6	
Ortho-k lens	7	
Lens case	6	
MPS bottle (Screw top)	0	
MPS (Solution)	0	
Total	45	28
6-month		
Conjunctival sacs	5	24
Eyelid	10	
Eyelashes	4	
Ortho-k lens	4	
Lens case	3	
MPS bottle (Screw top)	0	
MPS (Solution)	0	
Total	26	24
All	116	67

Colonization in ortho-k lens wearers at baseline: A total of 60 staphylococci (45 *S. aureus* and 15 CNS) were isolated from ortho-k subjects. *S. aureus* was present in seven (41.2%) conjunctival sacs, six (35.3%) eyelids, five (29.4%) eyelashes, and six (35.3%) spectacle frames. CNS was present in ten (58.8%) conjunctival sacs.

Colonization in ortho-k lens wearers at the 3-month visit: A total of 73 staphylococci (45 *S. aureus* and 28 CNS) were isolated from ortho-k subjects. *S. aureus* was present in samples from two (11.8%) conjunctival sacs, eight (47.1%) eyelids, three (17.6%) eyelashes, four (23.5%) ortho-k lenses, and three (17.6%) lens cases. There were no *S. aureus* isolated from samples of the screw tops of MPS bottle or from MPS. CNS was present in samples from 16 (94.1%) conjunctival sacs.

Colonization in ortho-k lens wearers at 6-month visit: A total of 50 staphylococci (26 *S. aureus* and 24 CNS) were isolated from ortho-k subjects. *S. aureus* was present in samples from two (13.3%) conjunctival sacs, six (40%) eyelids, four (26.7%) eyelashes, three (20%) contact lenses, and two (13.3%) lens cases. There were no *S. aureus* isolated from the screw tops of MPS bottles or the MPS. CNS was present in samples from 14 (93.3%) conjunctival sacs.

Table 4.3 Comparison of carriage of *S. aureus* and coagulase negative staphylococci over time.

Organism	Location	Group (No. of subjects)	Positive No. (%)	OR	<i>p</i> trend value	
<i>S. aureus</i>	Conjunctival sac	Baseline (17)	7 (41.2)			
		3-month (17)	2 (11.8)	0.19		
		6-month (15)	2 (13.3)	0.22	0.03	
	Eyelid	Baseline (17)	6 (35.3)			
		3-month (17)	8 (47.1)	1.63		
		6-month (15)	6 (40)	1.22	0.91	
	Eyelashes	Baseline (17)	5 (29.4)			
		3-month (17)	3 (17.6)	0.51		
		6-month (15)	4 (26.7)	0.87	0.68	
	Spectacle frames	Baseline (17)	6 (35.3)	NA		
	Ortho-k lens	3-month (17)	4 (23.5)			
		6-month (15)	3 (20)	0.81	0.81	
	Lens case	3-month (17)	3 (17.6)			
		6-month (15)	2 (13.3)	0.53	0.53	
CNS	Conjunctival sac	Baseline (17)	10 (58.8)			
		3-month (17)	16 (94.1)	11.2		
		6-month (15)	14 (93.3)	9.8	0.02	

NA, not applicable; OR: Odds Ratio; *p* value in bold indicates significance.

*Isolates of CNS only characterized from conjunctival sacs.

Carriage of *S. aureus* isolates in the conjunctival sac was significantly higher at baseline (41.2%) than at subsequent visits (11.8% at 3-month, and 13.3% at 6-month) (Odds Ratio (3-month) = 0.19; Odds Ratio (6-month) = 0.22; *p* trend value=0.03). *S. aureus* carriage rates on the eyelids and eyelashes at baseline were similar to those at subsequent visits. In addition, carriage rates of *S. aureus* on the ortho-k lenses and lens cases were similar at 3-month and 6-month visits. (Table 4.3)

The conjunctival sacs of 10 (58.8%) ortho-k subjects at baseline were colonized with CNS. In comparison, colonization was observed in 16 (94.1%) and 14 (93.3%) at 3-month and 6-month visits, which were significantly higher than that at baseline (Odds Ratio (3-month) = 11.2; Odds Ratio (6-month) = 9.8; *p* trend value=0.02).

4.3.3 Prevalence of QAC genes in *S. aureus* and coagulase negative staphylococci isolates from orthokeratology subjects over time

The overall frequency of QAC genes in *S. aureus* was lower than in CNS (Table 4.4), reaching significance for *qacA/B* (*S. aureus* 7.8%; CNS 17.9%; Odds Ratio = 2.59; Confidence Intervals: 1.03-6.53; *p* = 0.043) and *smr* (*S. aureus* 3.4%; CNS 11.9%; Odds Ratio = 3.79; Confidence Intervals: 1.1-13.13; *p* = 0.035). Only 5 (7.5%) CNS isolates carried *qacH* and no sample was positive for *qacG* or *qacJ*.

Table 4.4 Presence of *qacA/B*, *smr* and *qacH* in isolates of *S. aureus* and coagulase negative staphylococci from orthokeratology lens wearers over time.

Organism	Groups (No. of isolates)	<i>qacA/B</i>			<i>smr</i>			<i>qacH</i>		
		Positive No. (%)	OR	<i>p</i> trend value	Positive No. (%)	OR	<i>p</i> trend value	Positive No. (%)	OR	<i>p</i> trend value
<i>S. aureus</i>	Baseline (45)	2 (4.4)			2 (4.8)					
	3-month (45)	3 (6.7)	1.54		1 (2.2)	0.49				
	6-month (26)	4 (15.4)	3.91	0.18	1 (3.8)	0.86	0.58			
CNS	Baseline (15)	1 (6.7)			2 (13.3)			2 (13.3)		
	3-month (28)	5 (17.9)	3.04		2 (7.1)	0.5		1 (3.6)	0.24	
	6-month (24)	6 (25)	4.67	0.22	4 (16.7)	1.3	0.83	2 (8.3)	0.59	0.47
All	Baseline (60)	3 (5)			4 (6.7)			2 (3.3)		
	3-month (73)	8 (11)	2.34		3 (4.1)	0.6		1 (1.4)	0.4	
	6-month (50)	10 (20)	4.75	0.02	3 (6)	0.89	0.69	2 (4)	1.21	0.89

All *S. aureus* vs CNS:

qacA/B: *S. aureus* 7.8%; CNS 17.9%; OR 2.59; 95% CI 1.03-6.53; ***p*=0.043**.

smr: *S. aureus* 3.4%; CNS 11.9%; OR 3.79; 95% CI 1.1-13.13; ***p*=0.035**.

OR: Odds Ratio; CI: confidence intervals; *p* value in bold indicates significance.

Carriage rates of *qacA/B* in both *S. aureus* and CNS isolated from ortho-k subjects increased considerably over time. However, the difference over time, in *S. aureus* isolates ranged from 4.4% to 15.4% (Odds Ratio (3-month) = 1.54; Odds Ratio (6-month) = 3.91; p trend value=0.18) and in CNS ranged from 6.7% to 25% (Odds Ratio (3-month) = 3.04; Odds Ratio (6-month) = 4.67; p trend value=0.22), did not reach significance. Significant difference was observed in all staphylococcal isolates of 6.7% to 25% (Odds Ratio-3 month = 2.34; Odds Ratio -6 month = 4.75; p trend value=0.02). (Table 4.4)

The prevalence of *smr* and *qacH* were lower than *qacA/B* in both *S. aureus* and CNS and varied within a small range over time. Over the 6-month observation, the lowest rates of *smr* in *S. aureus* (2.2%) and CNS (7.1%) and *qacH* in CNS (3.6) were observed at the 3-month visit. There was no significant difference in the prevalence of *smr* and *qacH* in either *S. aureus* or CNS over the 6-month period (Table 4.4).

4.4 Discussion

Previous studies demonstrated that use of antiseptics may be selecting for increased presence of QAC genes in staphylococcal species (Zhang, *et al.* 2011, Zhang, *et al.* 2012) (See Section 1.14). In the previous chapter, the epidemiology of QAC genes in *S. aureus* and CNS isolated from ortho-k and spectacle wearers was compared. It was

found that long term use of antiseptics may contribute to changes of carriage of staphylococcus in the periorbital region and a higher presence of QACgenes among staphylococci species. However, only subjects who had been wearing ortho-k lenses for at least one year were recruited. There are no reports on the progress of this change, which may be gradual or may have appeared suddenly. In this study, the prevalence of QAC genes in staphylococci species isolated from ortho-k lens wearers was investigated over a six-month period.

4.4.1 Colonization with *S. aureus* over six months

S. aureus is a major cause of bacterial keratitis. Wearing of ortho-k lens led to changes of carriage of *S. aureus* in the periorbital region, which may contribute to a higher risk of ocular infection.

Carriage of *S. aureus* in the conjunctival sac was 41.2% at baseline (before wearing ortho-k lenses), which was similar to the result (40%) of control subjects in the previous study. This rate decreased remarkably after wearing ortho-k lenses (11.8% at 3-month visit and 13.3% at 6-month visit). However, among long term (over one year) ortho-k lens wearers a colonization rate of 21.7% was recorded. Several previous studies have investigated the impact of contact lens wear on the microbiota of conjunctival sac and observed the differences between contact lens wearers and

control subjects or before contact lens wear, but have shown conflicting results. Some researchers believed that pathogens, such as *S. aureus*, may be transferred into the conjunctival sac by the insertion of the contact lenses (Høvdig 1981, Fleiszig, *et al.* 1992, Cabrera, *et al.* 1996). However, the results of some other studies demonstrated that carriage of *S. aureus* in the conjunctival sac appeared to decrease or not altered by contact lens wear (Elander, *et al.* 1992, McClellan, *et al.* 1998, Boost, *et al.* 2005). Several studies have observed the normal microbiota of the conjunctival sac in children and found that carriage rates of *S. aureus* were lower than our results, ranging from 2.2-16.4% (Singer, *et al.* 1988, Fleiszig *et al.* 1992, Hautala, *et al.* 2008). In their studies, samples were directly sub-cultured onto agar plates and some organisms may be left on the swabs or be present in small numbers and be missed. Our swabs were enriched in BHI broth for 24 hours before sub-culture, which increased positive detection rates and allowed better estimation of the actual microbiota of the subjects. Our results demonstrated that there was a reduction in *S. aureus* colonization, which may be as a result of MPS remaining on the lens, which could kill or limit the growth of microbiota in the conjunctival sac. Under such a selective pressure, *S. aureus* may gradually adapt to this situation and more strains may be able to survive. These strains may contribute to a higher risk of ocular infection.

Carriage rates of *S. aureus* on the eyelids and eyelashes at baseline were similar to those of subsequent visits and were not obviously influenced by the wearing of ortho-

k lenses. In the previous study, carriage rates of *S. aureus* were higher on the eyelids and eyelashes of ortho-k subjects than those of spectacle controls, which suggested that organisms may have been transferred from the fingers to the skin of eyelids and eyelashes during ortho-k lens insertion and removal. In this study however, each subject was instructed on the use and care of the lens and lens care accessories at each visit. In general, new contact lens wearers have better compliance than long term wearer. Yung *et al.* (2007) demonstrated that a compliance enhancement strategy had a significant role in the improvement of compliance. The provision of individual instruction to each subject can contribute to good compliance, which may result in reduced effects of contact lens wear on the microbiota of eyelid and eyelashes. Carriage rates of *S. aureus* on the contact lenses and lens cases at the 3-month visit was similar to that of the 6-month visit, which may reflect stable rate of contamination of contact lens and lens case.

4.4.2 Colonization with coagulase negative staphylococci over six months

Compared with *S. aureus*, CNS are less pathogenic. Therefore, only CNS in the conjunctival sac was taken into account. Similar to the results in the earlier study, carriage rates of CNS in the conjunctival sac increased greatly after wearing ortho-k lens. However, many studies demonstrated that carriage rates of CNS were not obviously altered by insertion and removal of contact lenses and the use of MPS (Elander, *et al.* 1992, Cabrera, *et al.* 1996, McClellan, *et al.* 1998). As mentioned in

the discussion of the previous study, MPS left on the lens may destroy or inhibit the growth of CNS in the conjunctival sac. However, CNS was the most common organism in the conjunctival sac and showed a higher concentration than *S. aureus*. Some CNS may survive under the selective pressure of MPS and be detected after enrichment in BHI broth. In addition, some CNS may be transferred from the skin of finger into the conjunctival sac, leading to increased carriage rate in the conjunctival sac.

4.4.3 Prevalence of QAC genes in *S. aureus* and coagulase negative staphylococci isolated from orthokeratology subjects over time

The distribution of QAC genes in *S. aureus* and CNS has been investigated not only in clinical isolates, but also in strains isolated from the general population (Mayer, *et al.* 2001, Zhang, *et al.* 2011). Previous research demonstrated that QAC genes were more commonly detected in CNS than *S. aureus* (Zhang, *et al.* 2011, Zhang, *et al.* 2012). CNS is the most common organism in human normal microbiota, which may result in more opportunities to acquire QAC genes. In this study, significantly more *qacA/B* and *smr* were detected in CNS than *S. aureus*.

In the previous study (Chapter 3), the prevalence of QAC genes in Staphylococci were compared between ortho-k lens and spectacle wearers. Our study showed that

ortho-k lens wearers were more likely to be colonized with *S. aureus* and CNS harbouring QAC genes. Selective pressure from long term use of MPS may enhance the spread of QAC genes.

Over a 6-month observation period, prevalence of *qacA/B* in both *S. aureus* and CNS increased gradually with the linear trends of 1.8 and 1.48, respectively. Our results demonstrated that use of MPS may contribute to spread of *qacA/B* genes among staphylococcal species and this change appeared after wearing ortho-k lenses with related exposure to rigid lens MPS. The percentage of *S. aureus* positive for *qacA/B* at the 6-month visit was similar to that of subjects wearing ortho-k lens over one year, which demonstrated that the prevalence of *qacA/B* in *S. aureus* may be relatively stable after six months of lens wear. But, the prevalence of *qacA/B* in CNS at the 6-month visit was somewhat lower than the result (38.7%) in subjects who had worn ortho-k lenses for over one year, which suggested that the influence of selective pressure from long term use of MPS on acquiring *qacA/B* gene may be a long term process. To further investigate this trend and confirm this change, the observation period may need to be extended. The high carriage rate of *qacA/B* in CNS may contribute to the increasing incidence of ocular infections associated with CNS.

The prevalence of *smr* was much lower than that of *qacA/B* in both *S. aureus* and

CNS (Table 4.3). Most staphylococcus harbouring *smr* were clinical isolates, only a few studies have detected this gene in isolates from the community and general population and similar results in the prevalence of *smr* were detected in both *S. aureus* and CNS (Zhang, *et al.* 2011, Zhang, *et al.* 2012). Over a 6-month observation, the prevalence of *smr* in both *S. aureus* and CNS were quite stable, and no significant increase was found under the selective pressure of MPS, which may be due to low prevalence and small sample size. In the previous results reported in Chapter 3, similar results were found, with no significant difference in the prevalence of *smr* in either *S. aureus* or CNS between the two groups. Our results suggest that selective pressure from long term use of MPS showed less effect on the spread of *smr* than on *qacA/B*.

In this study, prevalence of *qac H* was low, only five CNS isolates carried *qacH* and no strains were positive for *qacG* or *qacJ*. These genes were initially detected in isolates from animals and occasionally reported in human isolates (Bjorland, *et al.* 2005, Zhang, *et al.* 2011, Ye, *et al.* 2012). A total of five CNS harbouring *qacH* were detected: two at the baseline, and one at the 3-month visit, and two at the 6-month visit. The prevalence of *qacH* in CNS was low and appeared not to be increased by exposure to MPS.

After 6-month lens wear, the prevalence of *qacA/B* in *S. aureus* appeared to reach a maximum level of 16% at which it remained stable. In contrast, frequency of *qacA/B* in CNS appeared to continue to increase after 6-month. However, this may be due to differences in the groups and be affected by relatively small sample size. In future studies, CNS isolated from other periocular sites should also be characterized and another large multi-center study may be required.

In summary, our results demonstrated that use of MPS led to selective pressure on the spread of QAC genes among *Staphylococcus* species in the eye. The percentage of strains harbouring QAC genes increased gradually over a 6-month observation period. As MPS are often diluted by saline, tap water or tears, reducing their disinfecting efficacy, more organisms harbouring QAC genes can survive, which may increase the chance of QAC genes spreading among *Staphylococcus* species. Therefore, good compliance and proper use of MPS play a significant role in the safe use of contact lenses to reduce the selective pressure for harbouring of QAC genes.

Paper published

Shi GS, Boost MV, Cho P. Prevalence of *qac* genes increases in staphylococcal isolates from orthokeratology lens wearers over initial six-month period of use. *European Journal of Clinical Microbiology & Infectious Diseases*. 2016 March 18. [Epub ahead of print]

CHAPTER 5

ANTIMICROBIAL SUSCEPTIBILITY OF STAPHYLOCOCCI HARBOURING QAC GENES

5.1 Introduction

Disinfectants, such as BAK and CHX, have been extensively used to prevent the spread of pathogens and control infection in hospitals and other health care settings (Gilbert, *et al.* 2005, Bragg, *et al.* 2014). However, with the widespread use of these products, there is a growing concern about the emergence of disinfectant-resistant microorganisms, notably in staphylococci, which will contribute to increasing difficulties in preventing infection. Extensive and inadequate use of these disinfectants could result in a selective pressure for survival of bacterial strains and the emergence and spread of QAC resistance genes (Hegstad, *et al.* 2010, Zhang, *et al.* 2011, Buffet-Bataillon, *et al.* 2012).

Several studies have demonstrated that antiseptic resistance genes in staphylococci are mainly plasmid-borne and confer reduced susceptibility to cationic antiseptic agents, including dyes (acriflavine, ethidium bromide), QACs, and biguanides by coding for efflux pumps, which reduce disinfectant concentration in the cell (Bjorland, *et al.*

2005, Smith, *et al.* 2008) (See Section 1.14). Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) have been used to observe changes in bacterial susceptibility to an antimicrobial agent in several studies and have shown elevated MIC and MBC in staphylococci harbouring QAC genes (Smith, *et al.* 2008, Vali, *et al.* 2008, Zhang, *et al.* 2011). In addition, several studies demonstrated that wide use of biocides might not only reduce effectiveness of biocides but also lead to some changes in susceptibilities to some antibiotics (Russell 2003, Vali, *et al.* 2008). QAC genes and antibiotic resistant determinants have been observed on the same plasmid (Sidhu, *et al.* 2001, Zmantar, *et al.* 2011). Studies have shown genetic linkage between QAC genes and several antibiotic resistance genes, such as *blaZ*, *ermC*, *dfrA*, and *aacA-aphD*, which resulted in resistance to several disinfectants together with β -lactam antibiotics, erythromycin, trimethoprim, and aminoglycosides (Sidhu, *et al.* 2001, Vali, *et al.* 2008, Zmantar, *et al.* 2011).

Antimicrobial susceptibility of *S. aureus* and CNS harbouring QAC genes has been investigated in several studies, but have involved mainly clinical isolates (Jaglic, *et al.* 2012, Costa, *et al.* 2013). In this study, the antimicrobial susceptibility of staphylococci harbouring QAC genes and the association between the presence of disinfectant resistance genes and antibiotic resistance determined in isolates was investigated. The strains were all isolated from asymptomatic ortho-k lens and spectacle wearers (See Chapter 3 or 4).

5.2 Methods

5.2.1 Bacterial strains

Two hundred and eighty two staphylococci (180 *S. aureus* and 102 CNS) isolated from ortho-k lens and spectacle wearers were tested in this study (Table 5.1). Among these isolates, 22 *S. aureus* and 38 CNS carried QAC genes. Forty *S. aureus* and forty CNS without QAC genes were randomly selected and used as controls in antiseptic susceptibility testing. All strains had been stored at -70 °C and were sub-cultured three times on nutrient agar (Oxoid, Basingstoke, UK) before use in this study.

Table 5.1. Summary of *Staphylococcus* tested in this study.

Presence of QAC genes	<i>S. aureus</i>	CNS
Negative	158	64
<i>qacA/B</i>	16	17
<i>Smr</i>	4	7
<i>qacA/B+smr</i>	2	4
<i>qacH</i>	--	5
<i>qacA/B+H</i>	--	3
<i>qacA/B+smr+H</i>	--	1
<i>smr+qacH</i>	--	1
Total	180	102

5.2.2 Antibiotic susceptibility

The antibiotic susceptibility of *S. aureus* and CNS isolates was tested using the disk diffusion method, following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009), except for fusidic acid, which followed methodology of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2010) (CLSI 2010, EUCAST 2011).

Freshly grown colonies were suspended into a 0.9% saline solution to prepare a 0.5 Mcfarland standard suspension. A sterile cotton swab was saturated by dipping into the inoculum suspension, rotated several times, and gently pressed on the inside wall of the saline tube to remove excess inoculum from the swab. The swab was streaked over the surface of Mueller-Hinton agar plate (Oxoid, Basingstoke, UK) three times, with the plate rotated approximately 60 ° each time to ensure even distribution of the inoculum. Finally, the agar rim was swept around using the swab.

Six antibiotic discs (Oxoid, Basingstoke, UK) were individually placed on the surface of Mueller-Hinton agar plate (90mm) using sterile forceps and the centre to centre distance was kept at over 24mm. Susceptibility to a total of 12 antibiotics was tested in this study: 11 for both *S. aureus* and CNS, including Penicillin G (10ug), Erythromycin (15ug), Gentamicin (10ug), Tetracycline (30ug), Ciprofloxacin (5ug),

Chloramphenicol (30ug), Clindamycin (2ug), Imipenem (10ug), Rifampicin (5ug), Trimethoprim/sulfamethoxazole, and Fusidic acid (10ug). Oxacillin (1ug) and Cefoxitin (30ug) were used to detect methicillin resistance in CNS and *S. aureus*, respectively.

The plates were placed in an incubator at 37°C for 24 hours. The zone diameters were automatically measured using the Mastscan Elite (Mast Group Ltd, Bootle, UK). Results were reported as susceptible, intermediate, and resistant, according to CLSI guidelines (CLSI, 2010), except for fusidic acid, which followed EUCAST (2010) clinical breakpoints. *S. aureus* (ATCC 25923) was used as a control strain.

5.2.3 Antiseptic susceptibility testing

MICs and MBCs of BAK and CHX were determined by the broth microdilution method with concentrations ranging from 0.5-64mg/L in Mueller-Hinton broth as described previously (Zhang, *et al.* 2011).

Briefly, three to five fresh colonies were inoculated into a bottle containing 10mL of Mueller-Hinton broth, and then the bottle was placed into the incubator at 37 °C (usually for 2-6 hours), until the broth reached a turbidity approximately equal to 0.5

McFarland standard (10^8 CFU/mL). An aliquot of 100 μ L of broth mixture was added into a bottle containing 9.9mL of Mueller-Hinton broth to achieve a concentration of 10^6 CFU/mL.

A 96-well microtiter plate was prepared and 90 μ L of Mueller-Hinton broth was added into the wells of row 2 to row 11. Into the first row of the microtiter plate, 180 μ L CHX (Sigma, St Louis, USA) or BAK (Sigma) at the concentration of 128mg/L was added and double dilution was performed to achieve serials concentration of 64, 32, 16, 8, 4, 2, 1, 0.5mg/L (rows 2 to 9). Inoculation of 10 μ l of bacterium broth (10^6 CFU/mL) into the wells (rows 2 to 10) was made to achieve a final concentration of 10^5 CFU/mL. Row 10, without any disinfectant, was regarded as a positive control. Row 11, which contained only 90 μ L of Mueller-Hinton broth was considered as a negative control.

The plate was incubated at 37°C for 24 hours. Turbidity indicated growth of the strains and the MIC was defined as the lowest concentration wells in which no growth was observed. An aliquot of 90 μ L of neutralizer (Dey-Engley broth, Sigma-Aldrich, St Louis, USA) was mixed with 10 μ L from each well with no growth observed and held for 10min. This mixture was then spread on a plate of nutrient agar. The agar plates were incubated at 37°C for 16-18 hours and viable microbial counts were

counted by an automated counter (aCOLyte Super Count, Synbiosis, Frederick, MD, USA). The concentration producing a 99.9% kill was regarded as the MBC (Fuursted *et al.*, 1997).

5.2.4 Statistical analysis

Statistical analyses were performed using SPSS system for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). The association between the presence of QAC genes and antibiotic resistance patterns was analyzed by Chi square statistic or Fisher's exact test. Results of MIC and MBC were compared by Mann-Whitney U-test. *P* value of 0.05 or below was considered as statistically significant.

5.3 Results

5.3.1 Antibiotic susceptibility in *S. aureus*

Results of antibiotic susceptibility testing are shown in Table 5.2. Resistance was frequent to penicillin and erythromycin in *S. aureus* with isolates displaying 79.4% and 54.4% resistance, respectively. Rates of resistance to clindamycin and tetracycline were 7.8% to 10.6%, respectively, while rates of resistance below 5% were observed to ceftiofur, chloramphenicol, gentamicin, ciprofloxacin, trimethoprim/sulphamethoxazole, imipenem, rifampicin and fusidic acid.

5.3.2 Antibiotics susceptibility in coagulase negative staphylococci

Resistance was more frequently observed in CNS to penicillin, erythromycin, oxacillin, chloramphenicol, tetracycline, clindamycin, and gentamicin at the rates of 67.6%, 33.3%, 32.4%, 20.6%, 19.6%, 14.7% and 14.7%, respectively. CNS resistant to ciprofloxacin and fusidic acid was 10 (9.8%). Rates of resistance to trimethoprim/sulphamethoxazole, imipenem, and rifampicin were lower than 5%.

5.3.3 Correlation between antibiotic resistance and presence of QAC genes in *S. aureus* and coagulase negative staphylococci

Resistance to most antibiotics was more likely in QAC gene positive isolates than in QAC gene negative isolates. However, differences did not reach significance for any of the antibiotics ($p > 0.05$). Significant association was noted between resistance to cefoxitin, ciprofloxacin, tetracycline, imipenem, and rifampicin in *S. aureus* isolates harbouring QAC genes ($p < 0.05$) (Table 5.2).

Table 5.2 Antimicrobial susceptibility patterns of *S. aureus* and coagulase negative staphylococci isolates.

Antibiotics	<i>S. aureus</i>					CNS				
	All (n=180)	QAC (+ve) (n=22)	QAC (-ve) (n=158)	OR	<i>p</i>	All (n=102)	QAC (+ve) (n=38)	QAC (-ve) (n=64)	OR	<i>p</i>
	R (%)	R (%)	R (%)			R (%)	R (%)	R (%)		
Oxacillin	N/A	N/A	N/A			33 (32.4)	16 (42.1)	17 (26.6)	2.01	0.11
Cefoxitin	3 (1.7)	2 (9.1)	1 (0.6)	15.7	0.03	NA	NA	NA		
Erythromycin	98 (54.4)	13 (59.1)	85 (53.8)	1.24	0.64	34 (33.3)	16 (42.1)	18 (28.1)	1.86	0.15
Chloramphenicol	8 (4.4)	2(9.1)	6 (3.8)	2.53	0.27	21 (20.6)	11 (28.9)	10 (15.6)	2.2	0.11
Clindamycin	14 (7.8)	3 (13.6)	11 (7)	2.11	0.28	15 (14.7)	9 (23.7)	6 (9.4)	3.0	0.055
Gentamicin	7 (3.9)	2 (9.1)	5 (3.2.)	3.06	0.2	15 (14.7)	8 (21.1)	7 (10.9)	2.17	0.17
Ciprofloxacin	2 (1.1)	2 (9.1)	0 (0.0)	>15.7	<0.03	10 (9.8)	5 (13.2)	5 (7.8)	1.79	0.39
Trimethoprim/ sulfamethoxazole	2 (1.1)	1 (4.5)	1 (0.6)	7.48	0.16	2 (2)	2 (5.3)	0 (0.0)	NA	
Tetracycline	19 (10.6)	7 (31.8)	12 (7.6)	5.68	0.002	20 (19.6)	10 (26.3)	10 (15.6)	1.93	0.19
Penicillin G	143 (79.4)	17 (77.3)	126 (79.7)	0.86	0.79	69 (67.6)	26 (68.4)	43 (67.2)	1.06	0.9
Imipenem	2 (1.1)	2 (9.1)	0 (0.0)	>15.7	<0.03	2 (2)	1 (2.6)	1 (1.6)	1.7	0.71
Rifampicin	4 (2.2)	2 (9.1)	2 (1.3)	7.8	0.05	4 (3.9)	2 (5.3)	2 (3.1)	1.72	0.59
Fusidic acid	4 (2.2)	1 (4.5)	3 (1.9)	2.46	0.44	10 (9.8)	6 (15.8)	4 (6.3)	2.81	0.13

P value in bold indicates significance; R: Resistant; NA: not applicable; +ve: positive; -ve: negative; OR: Odds Ratio

Table 5.3 Minimum inhibitory concentrations and minimum bactericidal concentrations of benzalkonium chloride for *S. aureus* and coagulase negative staphylococci with and without QAC genes.

Strains	QAC genes	MIC (mg/L)					MBC (mg/L)			
		No.	Range	MIC ₅₀	MIC ₉₀	<i>P</i>	Range	MBC ₅₀	MBC ₉₀	<i>p</i>
Benzalkonium Chloride										
<i>S. aureus</i>	—	40	0.5-4	1	2		0.5-8	2	4	
	<i>qacA/B</i>	16	0.5-4	2	4	<0.001	2-8	4	8	0.009
	<i>Smr</i>	4	0.5-4	2	4	0.09	4-8	4	8	0.01
	<i>qacA/B + smr</i>	2	2-4	2	4	0.032	4	4	4	0.23
	QAC positive	22	0.5-4	2	4	<0.001	4-8	4	8	0.001
CNS	—	40	0.5-2	1	2		0.5-4	2	4	
	<i>qacA/B</i>	17	0.5-8	2	4	0.002	2-8	4	8	0.009
	<i>Smr</i>	7	0.5-4	2	4	0.004	2-8	4	8	0.003
	<i>qacA/B + smr / qacH</i>	7	1-4	2	4	0.002	4-8	4	8	0.001
	<i>qacH</i>	5	2-4	4	4	<0.001	4-8	4	8	0.004
	<i>smr + qacH</i>	1	4				4			
	<i>qacA/B + smr + qacH</i>	1	2				4			
	QAC positive	38	0.5-8	2	4	<0.001	2-8	4	8	<0.001

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; *P* value in bold indicates significance

Table 5.4 Minimum inhibitory concentrations and minimum bactericidal concentrations of chlorhexidine digluconate for *S. aureus* and coagulase negative staphylococci with and without QAC genes.

Strains	QAC genes	MIC (mg/L)					MBC (mg/L)			
		No.	Range	MIC ₅₀	MIC ₉₀	<i>p</i>	Range	MBC ₅₀	MBC ₉₀	<i>p</i>
Chlorhexidine Digluconate										
<i>S. aureus</i>	—	40	0.5-1	0.5	1		0.5-2	1	2	
	<i>qacA/B</i>	16	0.5-2	1	2	0.001	1-8	2	4	<0.001
	<i>Smr</i>	4	0.5-1	0.5	1	0.29	1-2	1	2	0.14
	<i>qacA/B + smr</i>	2	1	1	1	0.024	2	2	2	0.034
	QAC positive	22	0.5-2	1	2	<0.001	1-8	2	4	<0.001
CNS	—	40	0.5-1	0.5	1		0.5-2	1	2	
	<i>qacA/B</i>	17	0.5-2	1	1	0.003	1-4	2	2	<0.001
	<i>Smr</i>	7	0.5-2	1	2	0.18	1-4	2	4	0.007
	<i>qacA/B + smr / qacH</i>	7	0.5-2	1	2	0.007	1-4	2	4	0.001
	<i>qacH</i>	5	0.5-2	1	2	0.028	1-4	2	4	0.014
	<i>smr + H</i>	1	1				2			
	<i>qacA/B + smr + qacH</i>	1	1				2			
	QAC positive	38	0.5-2	1	2	<0.001	1-4	2	4	<0.001

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; *P* value in bold indicates significance

5.3.4 Minimum inhibitory concentration and minimum bactericidal concentration of benzalkonium chloride and chlorhexidine digluconate

Tests were performed on all QAC gene positive isolates (22 *S. aureus* and 38 CNS). QAC gene negative isolates (40 *S. aureus* and 40 CNS) were also tested as controls. The MICs and MBCs of BAK and CHX for QAC gene positive strains were determined and are shown in Tables 5.3 and 5.4. Both MIC and MBC of BAK and CHX for *S. aureus* and CNS harbouring QAC genes were significantly higher than those of *S. aureus* and CNS without QAC genes ($p < 0.01$). Isolates harbouring individual QAC genes usually had higher MICs (MIC₅₀ and MIC₉₀) and MBCs (MBC₅₀ and MBC₉₀) to BAK and CHX. However, not all reached significance, probably due to the small sample size.

5.4 Discussion

This study focused on the antimicrobial susceptibility patterns of *Staphylococcus spp.* harbouring QAC genes to several antibiotics and two biocides and compared the antibiotic and disinfectant resistance between isolates with or without QAC genes.

5.4.1 Antibiotic resistance

Previous investigators have reported antibiotic resistance of isolates to be closely

associated with the presence of QAC genes. Gentamicin, macrolide, and other antibiotic resistance genes can coexist with QAC genes on the same plasmids. Resistance to several antibiotics (tetracycline, trimethoprim, and aminoglycosides) has frequently been reported in clinical isolates in association with QAC genes (Sidhu, *et al.* 2002, Vali, *et al.* 2008, Zhang, *et al.* 2011).

In our study, both *S. aureus* and CNS isolates carrying QAC genes (*qacA/B*, *smr* and *qacH*) were more resistant to tested antibiotics than those without QAC genes, which was consistent with several previous reports (Zhang, *et al.* 2011). But this difference did not always reach significance. Our results showed that only resistance to cefoxitin, ciprofloxacin, tetracycline, imipenem and rifampicin in *S. aureus* were significantly higher in isolates harbouring QAC genes. Differences in resistance between gene positive and negative strains for other antibiotics may become significant in a large scale study.

Several studies demonstrated that QAC genes were more commonly detected in methicillin-resistant strains (Zhang, *et al.* 2011, Alam, *et al.* 2003). Zhang *et al.* (2011) found that 50% MRSA and 16% methicillin-susceptible *Staphylococcus aureus* (MSSA) carried *qacA/B*. Alam *et al.* (2003) reported that prevalence of *qacA/B* was much higher in MRSA (32.6%) than MSSA (7.5%). It was suggested that strains

expressed resistance to methicillin, oxacillin and ceftiofur was commonly due to the presence of *mecA* gene that encoded penicillin-binding protein 2a (Ito, *et al.* 2001, Ma, *et al.* 2002, Zhang, *et al.* 2011). Although *mecA* which determines methicillin resistance is on the chromosome, its relationship with QAC genes is likely to be attributable to presence of the plasmid mediated gene in multi-drug resistant strains. Previous studies demonstrated that the β -lactam antibiotic resistance gene, *blaZ*, present on Tn552-like transposons, can produce β -lactamase (penicillinase) to hydrolyse the β -lactam ring of these antibiotics making the drug inactive. Genetic linkage between QAC genes and antibiotic resistance genes, including *blaZ*, has been reported in clinical and food industry isolates (Sidhu, *et al.* 2001, Sidhu, *et al.* 2002). Sidhu *et al.* (2001) showed that the multi-resistance plasmid, pST6, contained *qacB* and an incomplete β -lactamase transposon Tn552 that encoded the gene *blaZ* as well as *blaI* and *blaR* which control *blaZ* gene expression. Presence of QAC genes was also significantly associated with resistance to tetracycline. Sidhu *et al.* (2002) found that multi-resistance plasmid (pMS62) contained *qacA/B* and *blaZ* together with *tetK* mediating resistance to antiseptics, β -lactam antibiotic, and tetracycline. The determinant for tetracycline resistance was not characterized in the present study, but as *tetK* encodes for production of an efflux pump, it is likely that this gene is also present in our isolates (Schmitz, *et al.* 2001, Emaneini, *et al.* 2013).

S. aureus carrying QAC genes were also significantly more likely to display

resistance to ciprofloxacin, imipenem and rifampicin. Low level ciprofloxacin resistance is also attributable to presence of an efflux pump (Tanaka, *et al.* 2000). In the case of imipenem this is likely to be related to methicillin resistance as resistance to methicillin leads to resistance to all β -lactam agents (Fan, *et al.* 1986). Rifampicin resistance is usually associated with changes in *rpoB*, but presence of non-specific efflux pumps may also lead to removal of antibiotics entering the cell before reaching their target organelle (Aubry-Damon, *et al.* 1998, Costa, *et al.* 2013).

Genetic linkage between QAC genes and other antibiotic resistance genes has also been reported, such as *ermC* (erythromycin, clindamycin), *dfrA* (trimethoprim), and *aacA-aphD* (aminoglycoside) (Vali, *et al.* 2008, Coutinho Vde, *et al.* 2010, Zmantar, *et al.* 2011). However, the difference in the resistance to these antibiotics between strains carrying QAC genes and gene negative isolates did not reach significance. A larger sample size may be needed. Macrolide resistance in staphylococci is predominantly attributable to either presence of an efflux pump encoded by *msrA* or modification of the target site on the ribosome by *erm* genes (Weisblum 1995, Lina, *et al.* 1999, Coutinho Vde, *et al.* 2010, Zmantar, *et al.* 2011). Zmantar *et al.* (2011) demonstrated higher prevalence of *ermC* and *msrA* were observed in staphylococci species harbouring QAC genes. Chloramphenicol resistance genes in staphylococci are normally carried on several small plasmids with the size of 2.9-5.1 kb, such as pSK2, pSK5, pSK102, and pSK103 (Tennent, *et al.* 1986). Trimethoprim/

sulfamethoxazole resistance in staphylococci is mainly due to a chromosomal mutation in the *df_rB* gene or plasmid-mediated via *df_rA* gene. Mutation of endogenous dihydrofolate reductase generally confers low to moderate level resistance, whereas expression of horizontally acquired dihydrofolate reductase leads to high level resistance (Dale, *et al.* 1995). The genetic determinant contributing to aminoglycoside (gentamicin) resistance in staphylococci may be located on the chromosome or on a plasmid (Storrs, *et al.* 1988). The same determinant detected in the plasmid was sometimes located on the chromosoma, presumably due to transposition from the plasmid to the chromosome (Lyon, *et al.* 1984). Resistance to fusidic acid in staphylococci results from two mechanisms: designated *fusA*- type, spontaneous mutation in the chromosomal *fusA* gene; and *fusB*-type, in which plasmids mediate resistance via *fusB* genes. The *fusB* determinant was originally detected on a 21-kb plasmid pUB101. Now it can also be found in the chromosoma from staphylococci (Chen, *et al.* 2010, McLaws, *et al.* 2011). Transfer of resistance plasmids among species may contribute to their wide distribution among staphylococci species and explain less susceptibility to several antibiotics in strains harbouring QAC genes. In addition, several previous studies demonstrated that some antibiotic resistance genes (such as *aacA-aphD*, *df_rA*) encoding resistance to gentamicin, kanamycin, tobramycin and trimethoprim were located together with QAC genes on several plasmids (pST6, pMS62, pSK1, pSK4, and pSK41) and transposons (Tn552, Tn4001 and Tn4002) (Gillespie, *et al.* 1986, Berg, *et al.* 1998, Sidhu, *et al.* 2001). Other mechanism responsible for increased resistance to antibiotics may be outer membrane

changes, which lead to non-specific cross-resistance to antibiotics by efflux pumps encoded by QAC genes (Russell 2002, Russell 2003).

The association between QAC genes and antibiotics may increase the risk of antibiotic resistant infection. As non-compliance with correct routines of contact lens wear is the leading risk factor for MK, the increased potential for these infections to be caused by more resistant organisms which may be more difficult to treat is of concern.

5.4.2 Minimum inhibitory concentrations and minimum bactericidal concentrations of benzalkonium chloride and chlorhexidine digluconate

Extensive and inadequate use of disinfectants have commonly been observed in both clinical settings and the general environment, which may result in selective pressure for survival of bacterial strains and the emergence of QAC resistance genes, thus creating a potential risk for the control of infection in hospitals and other clinical settings. Several studies have demonstrated that there are links between bacterial resistance and use of biocides. In addition, the emergence of QAC resistance genes in *S. aureus* and CNS codes for increased resistance to a broad range of disinfectants (Zhang, *et al.* 2012, Bragg, *et al.* 2014).

Our results showed that MICs and MBCs of *S. aureus* and CNS carrying QAC genes to BAK and CHX were commonly higher than those of gene negative strains, which is consistent with expected changes. However, there were some differences between strains with different QAC genes. The six isolates (2 *S. aureus* and 9 CNS) harbouring two or more QAC genes showed higher MICs and MBCs than other isolates having one gene only, suggesting an additive effect. To avoid the influence of small sample size, more such isolates should be investigated. This effect has been noted in other studies (Zhang, *et al.* 2011).

The MICs and MBCs of the two antiseptics tested for all isolates were lower than actual concentrations recommended for general disinfection. However, disinfectants are used at lower concentrations in MPS to avoid toxicity to ocular tissues. In actual use for lens and lens accessories MPS are inevitably diluted by water, saline, and tears, which may further decrease the effectiveness of these disinfectants. The presence of organic materials, such as protein, is known to considerably reduce the effectiveness of disinfectants (Kawamura-Sato, *et al.* 2008, Stringfellow, *et al.* 2009). Kawamura-Sato *et al.* (2008) demonstrated that the MBCs of CHX and BAK for clinical *Acinetobacter* species rose from 32 to 256 and 512 µg/mL in the presence of 3% BSA, respectively. In addition, effectiveness of MPS can decrease during storage of the opened solutions (Leung, *et al.* 2004). Reduction in effectiveness can allow more strains harbouring QAC genes to survive and spread these genes among

staphylococcal species, which may explain the higher incidence of these resistance genes in ortho-k lens wearers. *In vitro* studies have shown that disinfectant resistance gene expression can be induced by exposure to sub-inhibitory concentrations of biocides (Smith, *et al.* 2008). Strains harbouring QAC genes may be more likely to survive the disinfection process and serve as a source of infection.

In summary, long term use of antiseptics may contribute to a higher presence of QAC genes among staphylococcal species isolated from ortho-k lens wearers. This may increase the risk of infection with an antibiotic resistant organism. Association with antibiotic resistance could make treatment of such infections more problematic. It is therefore important that disinfection of lenses is performed correctly, avoiding dilution of disinfection solution by topping up, re-use of disinfection containing organic materials or use of expired solutions, all of which may have reduced disinfecting power enhancing the survival of organisms carrying antiseptic resistance genes.

CHAPTER 6

EFFICACY OF MULTIPURPOSE SOLUTIONS FOR RIGID GAS PERMEABLE LENSES AGAINST STAPHYLOCOCCI HARBOURING QAC GENES.

6.1 Introduction

MK is the most serious complication associated with contact lens (Beljan, *et al.* 2013)wear . Various factors have been reported as being responsible for contact lens-related ocular infections. Microbial contamination of contact lens and lens cases is frequently detected in contact lens wearers and is a known risk factor for MK (Szcotka-Flynn, *et al.* 2010, Stapleton, *et al.* 2012). Staphylococci, which are the most common pathogens associated with MK are commonly isolated from contact lens and lens accessories of asymptomatic lens wearers (Yung, *et al.* 2007, Cho, *et al.* 2009). Contact lenses are considered as capable of transporting potential pathogens contaminating lens and lens accessories into the eyes. In published reports, the contamination rates of contact lenses, lens cases, and lens care solutions ranged have ranged from 9% to 84%, 25% to 87%, and 11% to 63%, respectively (Devonshire, *et al.* 1993, Hart, *et al.* 1993, Gray, *et al.* 1995, Lipener, *et al.* 1995, Rosenthal, *et al.* 1995, Midelfart, *et al.* 1996, Velasco, *et al.* 1996, Yung, *et al.* 2007, Cho, *et al.* 2009).

To reduce the incidence of ocular infections associated with contact lens wear, the use of contact lens disinfecting and cleaning systems are required. MPS are essential to inactivate microorganisms on the lenses and lens accessories (Boost, *et al.* 2006, Boost, *et al.* 2010, Mohammadinia, *et al.* 2012). Before being released to the market, each solution must be assessed for their antimicrobial efficacy according to certain criteria (ISO 14729 regulations). The stand-alone test challenges each product with five representative microorganisms, including two gram-negative bacteria (*P. aeruginosa* ATCC 9027 and *Serratia marcescens* ATCC 13880), one gram-positive bacteria (*S. aureus* ATCC 6538), one yeast (*Candida albicans* ATCC 10231), and one mold (*Fusarium solani* ATCC 36031). According to the FDA Guidance Document in 1997, a 3-log reduction of viable bacterial cells and a 1-log reduction of viable fungal cells must be achieved by each disinfecting solution under the recommended disinfection conditions. Several studies have examined the disinfecting efficiency of MPS for rigid gas permeable (RGP) lenses and found that all test solutions met the requirement of FDA standard (Food-and-Drug-Administration 1997) (Boost, *et al.* 2006, Boost, *et al.* 2010, Booranapong, *et al.* 2012, Kuzman, *et al.* 2013). (See Section 1.20.3)

With the widespread use of disinfectants, there is a growing concern about the emergence of disinfectant-resistant *Staphylococcus spp.* (Zhang, *et al.* 2011, Johnson, *et al.* 2013). The distribution of QAC genes in staphylococci has been investigated not

only in clinical isolates, but also in isolates from the general population (Mayer, *et al.* 2001, Zhang, *et al.* 2011). The previous results in Chapter 3 and Chapter 4 also demonstrated that long term use of antiseptics may contribute to a higher presence of QAC genes among staphylococci species isolated from ortho-k lens wearers. MICs and MBCs of strains harbouring QAC genes to BAK and CHX were higher than those of gene negative strains (Chapter 5), which suggested that strains harbouring QAC genes had reduced susceptibility to antiseptic agents and increasing the difficulties of preventing infection.

However, the use of the limited panel of the stand-alone test may not represent the actual efficiency of MPS. Several studies have been performed to test the efficacies of MPS against several non-FDA recommended strains, such as MRSA, *Streptococcus pneumoniae*, *H. influenzae*, *Acinetobacter*, and *Enterococcus* (Boost, *et al.* 2010).

However, there are no reports on the assessment of the efficiency of MPS against staphylococci harbouring QAC genes, which are commonly isolated from ortho-k lens wearers and may be more likely to survive the disinfection process and serve as a source of infection. The aim of this study was to investigate the efficacy of four MPS for RGP lenses against strains of staphylococci harbouring QAC genes isolated from ortho-k lens wearers.

6.2 Materials and methods

6.2.1 Contact lens solutions

Four brands of commonly used MPS for rigid contact lenses were used in this study: AMO TotalCare (Advanced medical optics, NSW, AUS), Boston Advance, Boston Simplus (Polymer Technology Corporation, Rochester, NY, USA), MeniCare Plus (Menicon Co. Ltd, Japan). All test solutions were purchased from local suppliers and were tested before their expiry date. Active ingredients of these solutions are shown in Table 6.1. The solutions were stored at room temperature (approximately 22°C) out of direct sunlight.

Table 6.1. Active ingredients of disinfecting solutions.

Type	MPS	MMRDT	Ingredients
A	AMO TotalCare	4 h	EDTA / PHMB 0.0001%
B	Boston Advance	4 h	CHX 0.003% / EDTA 0.05% / PHMB 0.0005%
C	Boston Simplus	4 h	CHX 0.003% / PHMB 0.0005%
D	MeniCare Plus	4 h	EDTA / PHMB 0.0005%

MMRDT: manufacturer's minimum recommended disinfection time

6.2.2 Microorganisms and culture conditions

Fifty eight staphylococci (22 *S. aureus* and 36 CNS) harbouring QAC genes isolated from ortho-k lens wearers were tested in this study (Table 6.2). Thirty *S. aureus* and thirty CNS without QAC genes isolated from ortho-k lens wearers were used as controls. All strains had been stored at -70 °C. They were sub-cultured three times on nutrient agar (Oxoid, Basingstoke, UK) before use in the stand-alone test.

Table 6.2. Summary of *Staphylococcus* tested in this study.

QAC gene present	<i>S. aureus</i>	CNS
Negative	30	30
<i>qacA/B</i>	16	17
<i>smr</i>	4	7
<i>qacA/B+smr</i>	2	4
<i>qacH</i>	--	5
<i>qacA/B+H</i>	--	3
<i>qacA/B+smr+qacH</i>	--	1
<i>smr+qacH</i>	--	1
Total	52	68

6.2.3 Preparation of bacterial inocula

A single bacterial colony from the agar plate was inoculated into 10mL Mueller-Hinton broth (Oxoid, Basingstoke, UK) and incubated at 37°C for 16-18 hours. The concentration of each inoculum reached was then adjusted to 10⁶CFU/mL with physiological saline.

6.2.4 Determination of antimicrobial activities of multipurpose solutions

A volume of 1.8mL of MPS was added into a polystyrene test tube and 0.2mL diluted inoculum was added into each tube. A final concentration of 10⁵CFU/mL was reached after mixing the solution. The resulting mixtures were incubated at room temperature for 4h according to the manufacturer's recommendations, as shown in Table 6.1. Two samples were removed from the mixture immediately after mixing and at 4h, respectively. For each sample, a 0.1mL aliquot was mixed with 9.9mL of neutralizer (Dey-Engley broth, Sigma-Aldrich, St Louis, USA) and 0.1mL of this diluted solution was spread on a nutrient agar plate. All plates were incubated at 37°C for 16h. Following incubation, microbial colonies were counted using an automated counter (aCOLyte Super Count, Synbiosis, Frederick, MD, USA). The procedure for each strain was performed in duplicate. The average number of CFU for each strain was recorded and log reduction was calculated according to the following formula:

$$\text{Log (viable count at 0 hour)} - \text{log (viable count at 4}^{\text{th}} \text{ hour)}$$

6.2.5 Determination of minimum inhibitory concentration

MICs of four MPS were determined by the broth microdilution method in 96 well microtitre plates with concentrations ranging from 1:1 to 1:512 in Mueller-Hinton broth as described previously (Zhang, *et al.* 2011).

Briefly, three to five fresh colonies were inoculated into a bottle containing 10 mL of Mueller-Hinton broth, and then the bottle was placed into the incubator at 37°C (usually for 2-6 hours), until the turbidity of the broth was approximately equal to 0.5 McFarland standard (approximately $1-2 \times 10^8$ CFU/mL). An aliquot of 100 µL of broth mixture was added into a bottle containing 9.9 mL of Mueller-Hinton broth and a concentration of 10^6 CFU/mL was achieved. Two-fold dilutions for each MPS were prepared and covered the concentration range from 1:1 to 1:512. An aliquot of 90 µL of MPS dilution was transferred to the wells on the microtitre plate and 10 µL diluted inoculum was added into each well. After mixing, each well contained 10^5 CFU/mL. The plates were incubated at 37°C for 16-18 hours and then examined for visible growth. Turbidity indicated growth of the microorganism and the MIC was defined as the lowest concentration of biocide with no visible growth. Wells containing bacteria but without MPS were regarded as positive controls. The wells containing only 1:1 MPS were considered as negative controls.

6.2.6 Determination of minimum bactericidal concentration

A sample of 10 μ L from each well with no growth observed was mixed with 90 μ L of neutralizer (Dey-Engley broth, Sigma-Aldrich, St Louis, USA) and held for 10min.

This mixture was then spread on a nutrient agar plate. The agar plates were incubated at 37°C for 16-18 hours and MBCs were recorded as the lowest concentration of antiseptic that killed 99.9% of the original numbers of bacteria.

6.2.7 Statistical analysis

Statistical analyses were performed using SPSS system for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). Kruskal-Wallis test was used to compare the differences between groups for one condition, Mann-Whitney U-test was used to examine the differences between QAC gene negative strains and groups with different QAC genes for one solution. The associations between the presence of QAC genes and the number of isolates with less than 3-log reduction were analyzed by Chi square statistic or Fisher's exact test. *P* value of 0.05 or below was considered as statistically significant.

6.3 Results

6.3.1 Efficacy of multipurpose solutions for rigid lenses on *S. aureus* and coagulase negative staphylococci harbouring QAC genes

Twenty two *S. aureus* harbouring QAC genes were challenged with four MPS by the stand-alone test. Efficacy in log reduction of four MPS for QAC gene positive strains are shown in Table 6.3. All tested MPS displayed lower efficacy and wider ranges of log reduction against *S. aureus* harbouring QAC genes than those of *S. aureus* without QAC genes. However, this only reached significance for MPS-A ($p = 0.002$) and MPS-C ($p = 0.03$). Significant differences were found in *S. aureus* harbouring *qacA/B* ($p = 0.03$) and *smr* ($p = 0.02$) for MPS-A. Presence of *qacA/B+smr* resulted in lower log reductions for MPS-A ($p = 0.02$), MPS-B ($p = 0.02$) and MPS-C ($p = 0.04$). There were no significant changes in log reductions associated with presence of QAC genes in MPS-D. The distribution of log reductions of the four MPS for *S. aureus* are shown in Figure 6.1. All MPS achieved a 3-log reduction for over 90% of gene negative *S. aureus*. Except for MPS-D, no MPS achieved a 3-log reduction in *S. aureus* harbouring *qacA/B* and *smr*. Viability of only one *S. aureus* was not reduced by 3-log reductions by MPS-D, which much fewer the number not adequately effected by MPS-A (14 *S. aureus*), B (7 *S. aureus*) and C (15 *S. aureus*). MPS-A and MPS-C were unable to achieve even a 1-log reduction in some *S. aureus* isolates harbouring *qacA/B* or *smr*.

Table 6.3 Log reductions of of four multipurpose solutions for *S. aureus* and coagulase negative staphylococci with and without QAC genes.

QAC genes	No.	LR (MPS-A)			LR (MPS-B)			LR (MPS-C)			LR (MPS-D)		
		Range	Median	<i>p</i>	Range	Median	<i>p</i>	Range	Median	<i>p</i>	Range	Median	<i>p</i>
<i>S. aureus</i>													
—	30	2.81-5.3	5.18		2.8-5.3	5.18		2.19-5.3	5.15		5.08-5.3	5.18	
<i>qacA/B</i>	16	0.9-5.26	2.9	0.03	1.56-5.26	5.11	0.52	0.74-5.26	3.11	0.17	2.64-5.26	5.18	0.7
<i>smr</i>	4	0.78-5.18	1.63	0.02	2.42-5.26	5.08	0.42	1.19-5.18	2.35	0.06	3.32-5.26	5.08	0.33
<i>qacA/B + smr</i>	2	1.13-1.88	1.13	0.02	2.38-2.73	2.38	0.02	1.26-2.51	1.26	0.04	5.08-5.2	5.08	0.53
QAC positive	22	0.78-5.3	2.18	0.002	1.56-5.3	5.11	0.18	0.74-5.3	2.6	0.03	2.64-5.3	5.18	0.422
CNS													
—	30	1.75-5.28	5.15		2.56-5.28	5.15		2.11-5.28	5.15		5.04-5.28	5.15	
<i>qacA/B</i>	17	1.46-5.28	5.2	0.24	1.19-5.28	5.2	0.09	0.86-5.28	5.2	0.5	1.4-5.28	5.2	0.15
<i>smr</i>	7	2.18-5.26	5.08	0.3	3.26-5.26	5.11	0.13	2.41-5.26	5.08	0.07	2.95-5.26	5.11	0.07
<i>qacH</i>	5	2.36-5.26	5.23	0.51	3.23-5.26	5.2	0.63	2.73-5.26	5.23	0.51	3.2-5.26	5.23	0.66
≥ 2 genes	9	2.07-5.23	5.11	0.11	2.85-5.23	5.11	0.25	2.2-5.23	5.11	0.17	3.15-5.23	5.11	0.24
QAC positive	38	1.46-5.28	5.11	0.66	1.19-5.28	5.18	0.87	0.86-5.28	5.11	0.6	1.4-5.28	5.18	0.93

LR: log reduction of an initial approximate 5-log concentration of bacteria; *P* value in bold indicates significant difference between gene positive organisms and strains not harbouring QAC genes; MPS-A: AMO TotalCare; MPS-B: Boston Advance; MPS-C: Boston Simplus; MPS-D: MeniCare Plus

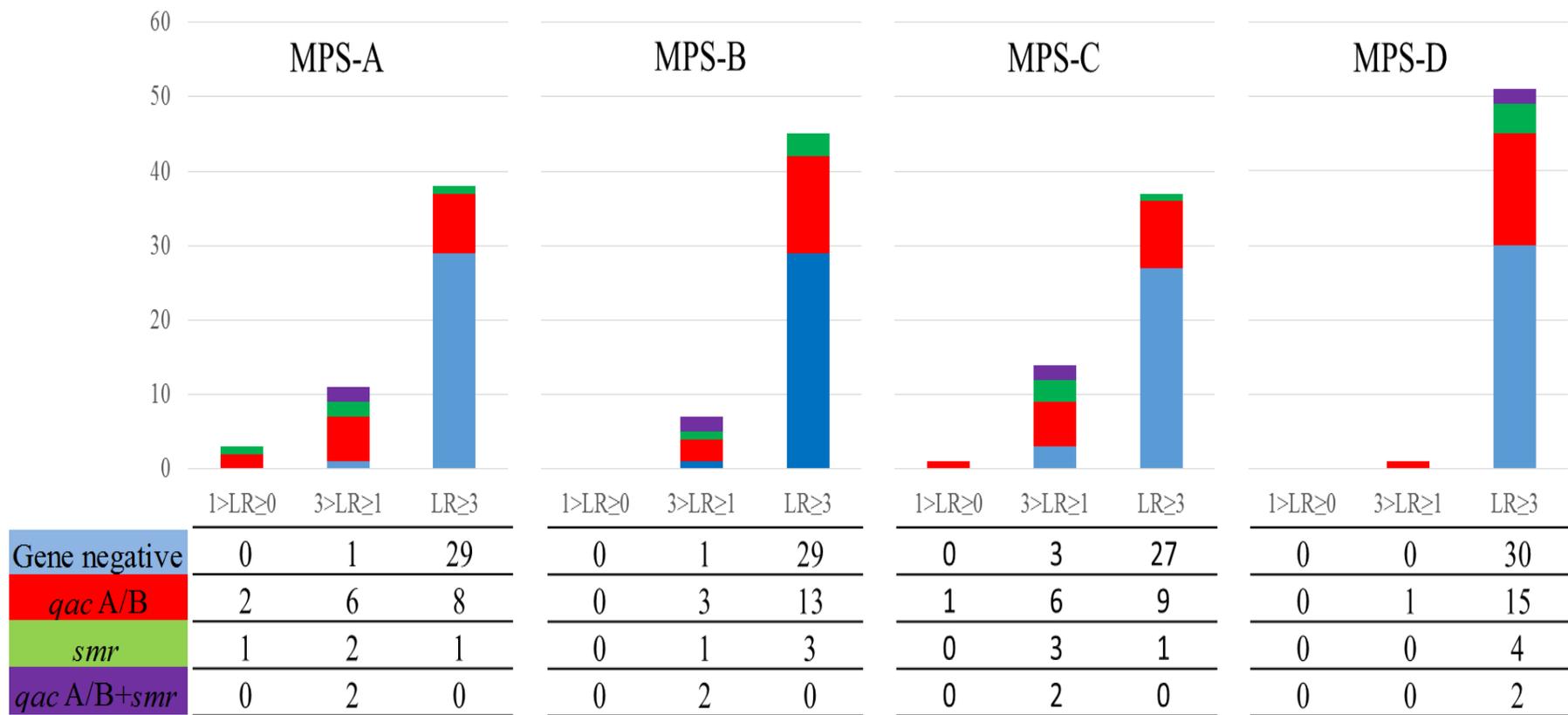


Figure 6.1 Distribution of log reduction of four multipurpose solutions for *S. aureus* isolates. (MPS-A: AMO TotalCare; MPS-B: Boston Advance; MPS-C: Boston Simplus; MPS-D: MeniCare Plus)

All four MPS challenged with 38 CNS harbouring different QAC genes showed a lower log reduction with wider ranges than to gene negative CNS. However, similar median log reductions were achieved for CNS with or without QAC genes. The distribution of log reductions of the four MPS for CNS are shown in Figure 6.2. A 4-log or greater log reduction was achieved for over 90% of gene negative CNS. Only 1 CNS harbouring *qacA/B* was not reduced by 1-log reduction following exposure to MPS-C.

The numbers and percentages of strains with less than 3-log reduction for tested MPS are shown in Table 6.4. Both *S. aureus* and CNS isolates with less than 3-log reduction to each MPS were more commonly detected in isolates harbouring QAC genes than gene negative isolates. However, significant differences were only found in *S. aureus* for MPS-A ($p = 0.0007$), B ($p = 0.03$) and C ($p = 0.001$) and no difference was detected in CNS and for MPS-D. Overall, inability to achieve 3-log reduction was more common for MPS tested with *S. aureus* than CNS, although MPS-D failed to achieve this reduction in three CNS but only one *S. aureus* isolate.

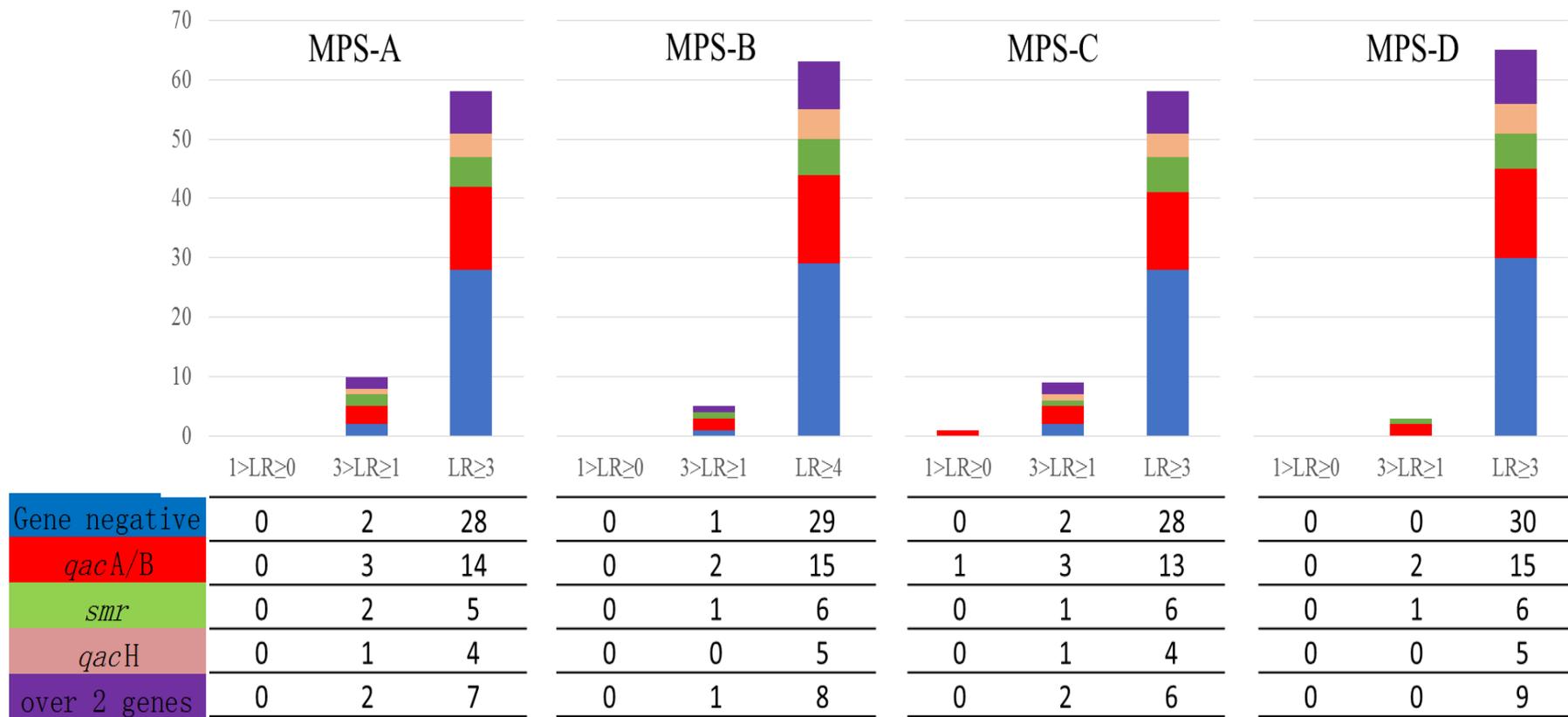


Figure 6.2 Distribution of log reduction of four multipurpose solutions for coagulase negative staphylococci isolates. (MPS-A: AMO TotalCare; MPS-B: Boston Advance; MPS-C: Boston Simplus; MPS-D: MeniCare Plus)

Table 6.4 Numbers and percentages of strains with less than 3-log reduction for four multipurpose solutions.

QAC genes	No	MPS-A			MPS-B			MPS-C			MPS-D		
		Number not reduced by 3 LR (%)	OR	<i>p</i>	Number not reduced by 3 LR (%)	OR	<i>p</i>	Number not reduced by 3 LR (%)	OR	<i>p</i>	Number not reduced by 3 LR (%)	OR	<i>p</i>
<i>S. aureus</i>													
—	30	1 (3.3)			1 (3.3)			3 (10)			0 (0)		
<i>qacA/B</i>	16	8 (50)	29	0.003	3 (18.8)	6.69	0.11	7 (43.8)	7	0.01	1 (6.3)	NA	
<i>smr</i>	4	3 (75)	29	0.01	1 (25)	9.67	0.14	3 (75)	27	0.01	0 (0)	NA	
<i>qacA/B + smr</i>	2	2 (100)	>58	<0.01	2 (100)	>58	<0.01	2 (100)	>18	<0.03	0 (0)	NA	
QAC positive	22	13 (59.1)	41.9	0.0007	6 (27.3)	10.9	0.03	12 (54.5)	10.8	0.001	1 (4.5)	NA	
CNS													
—	30	2 (6.7)			1 (3.3)			2 (6.7)			0 (0)		
<i>qacA/B</i>	17	3 (17.6)	3	0.26	2 (11.8)	3.87	0.29	4 (23.5)	4.31	0.12	2 (11.8)	NA	
<i>smr</i>	7	2 (28.6)	5.6	0.12	0 (0)	NA		1 (14.3)	2.33	0.52	1 (14.3)	NA	
<i>qacH</i>	5	1 (20)	3.5	0.35	0 (0)	NA		1 (20)	3.5	0.35	0 (0)	NA	
≥ 2 genes	9	2 (22.2)	4	0.2	1 (11.1)	3.63	0.38	2 (22.2)	4	0.2	0 (0)	NA	
QAC positive	38	8 (21.1)	3.73	0.11	3 (7.9)	1.2	0.84	8 (21.1)	3.73	0.11	3 (7.9)	NA	

OR: Odds Ratio; NA: not applicable; *P* value in bold indicates significance.

(MPS-A: AMO TotalCare; MPS-B: Boston Advance; MPS-C: Boston Simplus; MPS-D: MeniCare Plus)

Table 6.5. Overall minimum inhibitory concentrations and minimum bactericidal concentrations of four multipurpose solutions for *S. aureus* and coagulase negative staphylococci with and without QAC genes.

Strains	QAC genes	No.	MIC			MBC		
			Range	MIC50	<i>P</i> -value	Range	MBC50	<i>P</i> -value
MPS-A								
CNS	-ve	30	0.06-1	0.25		0.13-1	0.25	
	+ve	38	0.12-1	0.25	<0.001	0.13-1	0.5	<0.001
<i>S.aureus</i>	-ve	30	0.06-1	0.5		0.13-1	0.5	
	+ve	22	0.25-1	0.5	0.016	0.5-1	1	0.006
MPS-B								
CNS	-ve	30	0.06-0.13	0.06		0.13	0.13	
	+ve	38	0.06-0.25	0.13	<0.001	0.13-0.25	0.13	0.009
<i>S.aureus</i>	-ve	30	0.06-1	0.06		0.13-1	0.13	
	+ve	22	0.06-0.25	0.13	0.008	0.13-0.25	0.13	0.255
MPS-C								
CNS	-ve	30	0.06-0.25	0.06		0.13-0.5	0.13	
	+ve	38	0.06-0.25	0.06	0.001	0.13-0.25	0.13	0.128
<i>S.aureus</i>	-ve	30	0.06-0.5	0.06		0.13-1	0.13	
	+ve	22	0.06-1	0.13	0.052	0.13-1	0.25	0.012
MPS-D								
CNS	-ve	30	0.06-0.25	0.06		0.06-0.5	0.13	
	+ve	38	0.06-0.25	0.13	<0.001	0.13-0.25	0.13	0.015
<i>S.aureus</i>	-ve	30	0.06-0.25	0.06		0.13-0.5	0.13	
	+ve	22	0.06-0.5	0.13	0.011	0.13-1	0.25	0.017

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; *P* value in bold indicates significance.

(MPS-A: AMO TotalCare; MPS-B: Boston Advance; MPS-C: Boston Simplus; MPS-D: MeniCare Plus)

6.3.2 Minimum inhibitory concentrations and minimum bactericidal concentrations of four multipurpose solutions

The MICs and MBCs of the four MPS for strains harbouring QAC genes are shown in Table 6.5. In most cases, strains carrying QAC genes had higher MICs and MBCs for all four MPS than gene negative strains, reaching significance except for MBCs to MPS-B for *S. aureus* and to MPS-C for CNS.

6.4 Discussion

The previous studies (Chapters 3 and 4) have suggested that due to more exposure to of antiseptics, there was a higher presence of QAC genes among staphylococci isolated from ortho-k lens wearers than spectacle wearers. Evidence from other studies have also demonstrated that strains harbouring QAC genes had higher tolerance to a broad range of disinfectants, including QACs, dyes and biguanides (McDonnell, *et al.* 1999, Zhang, *et al.* 2011) (See Section 1.14). CHX and PHMB are QAC compounds used in several MPS for disinfection of contact lenses (Table 1.7). Therefore, there is an increased concern on the disinfecting efficacy of these MPS.

Although standardized testing of contact lens solutions is required before approval, only a limited number of organisms are involved in the stand-alone test (Food-and-Drug-Administration 1997). As mentioned before, in the stand-alone test the use of the limited panel may not be representative of the levels of biocide resistance displayed by organisms present in conjunctival sac, on the lenses or in the lens cases

of patients. The presence of strains carrying QAC genes may contribute to potential risks for ocular infections and it is important to investigate the efficacy of MPS against strains harbouring QAC genes.

6.4.1 Efficacy of multipurpose solutions for rigid lenses on *S. aureus* and coagulase negative staphylococci harbouring QAC genes

The results revealed that QAC gene positive isolates had lower log reductions to all four MPS than QAC gene negative isolates. This confirmed previous observations and our finding that reduced biocide susceptibility in staphylococci is associated with QAC genes (Smith, *et al.* 2008, Furi, *et al.* 2013). Staphylococci harbouring QAC genes had reduced susceptibility to MPS, which may enhance their survival on contact lenses and in storage cases. But some differences were observed in the susceptibility to MPS between *S. aureus* and CNS harbouring QAC genes. Compared with *S. aureus* harbouring QAC genes, all MPS achieved at least a 3-log reduction in a higher proportion of CNS harbouring QAC genes, suggesting that CNS harbouring QAC genes have a higher susceptibility to the tested MPS. Similar median log reductions to all MPS tested were achieved for CNS with and without QAC genes, which indicated that presence of QAC genes contributed less to survival of CNS than *S. aureus*.

QAC genes encode multidrug efflux pumps, which are membrane proteins capable of exporting many biocides out of the cells (Russell 2002, Costa, *et al.* 2013).

Generally, the presence of two QAC genes led to lower log reduction than isolates having only one QAC which may be due to additive effects of efflux pumps. This

additive effects of efflux pumps has been noted in other reports, which showed that isolates harbouring both *qacA/B* and *smr* had much higher MIC₅₀ and MBC₅₀ to several biocides, such as BAK and CHX (Zhang, *et al.* 2011). In this study, all *S. aureus* harbouring *qacA/B* + *smr* were not reduced by 3-logs by MPS-A, B and C. However, this change was not observed in CNS harbouring more than one gene, which may be due to its lower resistance to biocides and this observation needs to be further investigated.

In this study, the proportion of isolates with less than 3-log reduction was higher in strains harbouring QAC genes, but the difference reached significance only for *S. aureus* for MPS-A, B, and C (Table 6.4). MPS-D only failed to achieve a 3-log reduction against one *S. aureus* and three CNS compared with much higher proportions of disinfection failure noted for the other three disinfecting solutions (Table 6.4). MPS-D showed much higher antimicrobial efficacy than the other MPS, which was similar to previous results (Boost, *et al.* 2006). This solution showed a higher median of log reduction of *S. aureus*, especially QAC positive strains, than others MPS. Even though some MPS have same disinfecting agents at similar concentration, they may have different disinfecting efficacy. Different MPS, even with the same active ingredient, can show different antimicrobial efficacy (Boost, *et al.* 2006). In this study, the active ingredients of the four tested solutions were CHX and/or PHMB, which are all cationic biocides and widely used in clinical settings. Disinfecting efficacy is likely to be associated with other components (such as EDTA) in MPS which may affect surfactant power or enhance its activity.

Several studies have also tested the efficacy of MPS against some important ocular pathogens which were not included in the panel of stand-alone test. It was found that clinical isolates were commonly less susceptible to the tested MPS than FDA recommended strains, but all tested MPS were found to be effective in destroying these pathogens, including some *Acinetobacter* carrying QAC genes, and able to meet the 3-log reduction similar to the requirements of the stand-alone test (Boost, *et al.* 2010, Boost, *et al.* 2014). However, our results demonstrated that not all isolates were able to achieve a 3-log reduction in numbers for the four MPS within the manufacturer's recommended disinfection times. For both MPS-A and C, more than half *S. aureus* harbouring QAC genes survived after 4h disinfection. In actual application, more strains may survive due to non-compliance. The behavior of contact lens wearers have been evaluated in several studies and up to 91% of contact lens wearers had some degree of non-compliance when handling their lenses (Claydon, *et al.* 1994, de Oliveira, *et al.* 2003, Bui, *et al.* 2010). Non-compliance with lens care solutions, such as not using the recommended solution, re-use of solution, and using expired solutions, were commonly observed in a large proportion of contact lens wearers (Yung, *et al.* 2007, Robertson, *et al.* 2011). (See Section 1.18.2) This may allow more strains, especially strains harbouring QAC genes, to survive due to inadequate disinfection.

Overall, the presence of QAC genes in staphylococci contributed to decreased susceptibility to MPS, possibly allowing a higher chance to survive after disinfection. This may increase the potential risks of ocular infection, especially in children using ortho-k.

6.4.2 Minimum inhibitory concentrations and minimum bactericidal concentrations of four multipurpose solutions

In most cases, isolates with QAC genes had significantly higher MICs and MBCs to all four MPS; only the MBCs to MPS-B in *S. aureus* and to MPS-C in CNS did not reach significant difference. These results demonstrated that presence of QAC genes resulted in the reduction of the efficacies of MPS.

Commonly MPS for rigid lenses provide better disinfecting capability than those for soft lenses, as the concentration of the disinfecting agents tend to be higher, which suggests that more strains may survive after disinfecting by MPS for soft contact lenses and be potential pathogens for ocular infection. These solutions should also be evaluated against QAC positive isolates.

MIC and MBC were determined after 24 h incubation using a parallel method for determining MICs and MBCs of other antimicrobial agents. However, manufacturers recommend far shorter exposure times for disinfection. It may be useful to determine MICs and MBCs for the shorter disinfecting periods. As these parameters were being measured for solutions and not for the disinfecting compounds they are not strictly comparable to results obtained for individual agents. Nevertheless, they do give a strong indication of the level of activity of these solutions against staphylococci with and without QAC genes. It may be useful to evaluate the disinfecting efficacy of each MPS by both stand-alone test and determination of MIC and MBC. In addition, it must be remembered that MPS in actual use for disinfection of lenses are frequently

diluted by water, saline, or other solutions which will decrease the effects of the MPS.

If organisms do survive disinfection due to presence of QAC genes and are transferred to the conjunctival sac, they may be potential risks for ocular infection. Such infections may be more difficult to treat due to cross resistance of such strains to antibiotics. Transfer of MPS to the eye should be avoided as this provides low level exposure and selection for disinfectant tolerant strains. This can be achieved by rinsing lenses in sterile saline before insertion.

In summary, this study is of considerable clinical importance, as the staphylococci tested were all isolated from asymptomatic ortho-k lens and spectacle wearers. The results demonstrated that the presence of QAC genes in staphylococci significantly decreased their susceptibility to MPS for rigid lenses. Strains harbouring QAC genes may be more likely to survive the disinfection process and serve as a source of infection. For safe use of contact lenses, it may be necessary to involve some representative clinically isolates into the panel of the stand-alone test.

Paper published

Shi GS, Boost MV, Cho P. Does the presence of QAC genes in staphylococci affect the efficacy of disinfecting solutions used by orthokeratology lens wearers? *The British Journal of Ophthalmology*. 2016 May;100(5):708-12. doi: 10.1136/bjophthalmol-2015-307811. Epub 2015 Dec 30.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Introduction

The overall purpose of this study was to investigate the prevalence of QAC genes in *Staphylococcus* isolated from selected children (ortho-k lens and spectacle wearers) in Hong Kong and the effects of the period of use on that prevalence. In addition, determining the antibiotic susceptibility of QAC positive strains would help to confirm if harbouring these genes increases antibiotic resistance. These findings provide useful information for safe use of contact lenses. In this chapter, the conclusions of the results of this study are summarized. In accordance with the findings in this research, relevant recommendations are identified and further, the potential for utilization and clinical applications are also indicated. In addition, the limitations of this research work are identified and suggestions for further work are included in this chapter.

7.2 Conclusions and recommendations

The main issues addressed in this research were reviewed and the conclusions and relevant recommendations on these issues are summarized in this section.

7.2.1 Prevalence of QAC genes in *Staphylococcus* isolated from selected children in Hong Kong

With the wide use of disinfectants, there is an increasing concern worldwide about the prevalence of microorganisms harbouring of QAC genes, notably in staphylococci (Jaglic, *et al.* 2012, Johnson, *et al.* 2013). The use of biocides may lead to selective pressures for the survival of the bacteria and be one of the main reasons for the emergence of resistance genes (Hegstad, *et al.* 2010, Zhang, *et al.* 2011, Buffet-Bataillon, *et al.* 2012). In this research, a cross-sectional study and a longitudinal study were performed to determine the prevalence of *Staphylococcus* carrying QAC genes in selected children and changes occurring after wearing ortho-k lenses over a 6-month period of observation.

The prevalence of QAC genes in staphylococci has been investigated in previous studies, but most focused on clinical isolates (Mayer, *et al.* 2001, Sidhu, *et al.* 2002, Smith, *et al.* 2008, Longtin, *et al.* 2011) and only a few studies reported the prevalence of QAC genes in staphylococci isolated from the community and the general population (Zhang, *et al.* 2011, Zhang, *et al.* 2012). Staphylococcal species carrying QAC genes were found to be widespread on automated teller machines, which may act as a common source in the community for the spread of organisms harbouring QAC genes (Zhang, *et al.* 2012). General population may acquire strains carrying QAC genes from public environments.

Until now, there was no relevant report on the presence of QAC genes in isolates from

contact lens wearers. In the cross-sectional study, twenty three ortho-k lens wearers (at least one year lens wear history) and twenty spectacle wearers (no contact lens wear history) were included. This study concluded that QAC genes were also commonly detected in staphylococci isolates from both ortho-k lens and spectacles wearers, but ortho-k lens wearers had a significantly higher risk of carrying staphylococci harbouring QAC genes than spectacle wearers.

The results of this study suggested that long term use of MPS may select for the carriage organisms harbouring QAC genes. However, there were no longitudinal studies on the process of building up of the presence of disinfectant-resistance genes under the selective pressure of long term use of MPS. With this in mind, a 6-month longitudinal study on the prevalence of *Staphylococcus* carrying QAC genes was conducted to follow their presence in children using ortho-k lenses from commencement of their use. The results of this longitudinal study suggested that the risk of isolates harbouring QAC genes increased gradually with time in ortho-k lens wearers.

From the results of these two studies, we concluded that long term use of antiseptics contributed to a higher presence of QAC genes among staphylococci, which increased gradually over time. However, it does not mean that the prevalence will continue to increase. According the findings, we surmised that the prevalence of QAC genes in staphylococci after a period of increase may reach a plateau and then keep at a relatively stable level. Presence of additional genes can contribute to a relative

reduction in fitness of bacteria and thus reduce their growth rate at times when there is no advantage in presence of the gene i.e. when there is no disinfectant challenge. These differences in growth rate can allow for persistence of gene-negative strains (Klumpp, *et al.* 2009, Klumpp, *et al.* 2014).

In actual use, MPS is frequently diluted with tap water, saline or tears, which will reduce its disinfecting efficacy (Cho, *et al.* 2009, Mohammadinia, *et al.* 2012).

Bacteria carrying QAC genes may have more chance to survive and spread these genes among staphylococcal species (Smith, *et al.* 2008, Hegstad, *et al.* 2010).

Therefore, good compliance and proper use of MPS play a significant role in the safe use of contact lenses and reduce the selective pressure on the presence of QAC genes.

It may be possible to reduce selective pressure from disinfectants by use of solutions not selecting for QAC gene presence, such as hydrogen peroxide system, but this needs to be further investigated.

For nearly a decade, more children have chosen contact lenses to correct refractive errors and/or control the development of myopia (Cho, *et al.* 2005, Chen, *et al.* 2009, Chen, *et al.* 2013). Their immune systems may be less developed than the adults.

Higher carriage rate of isolates harbouring QAC genes may lead to higher risk of infectious diseases. Regular follow-up to examining the ocular health and close monitoring of the presence of isolates carrying QAC genes may be necessary for these children.

In previous studies, the prevalence of *qacA/B* and *smr* were more frequently investigated and were significantly higher than those of *qacG*, *H*, and *J* (Zhang, *et al.* 2012, Costa, *et al.* 2013). These genes are more typical in animal or food isolates and so are less likely to be isolated from human subjects not in contact with these sources (Heir, *et al.* 1999, Bjorland, *et al.* 2005, Correa, *et al.* 2008, Vali, *et al.* 2008, Ye, *et al.* 2012). Neither *qacG* nor *J* were found in any of the ocular isolates, and *qacH* was detected in only ten CNS isolated from both ortho-k lens and spectacle wearers. In this research, in contrast to *qacA/B* and *smr*, no obvious relationship was found between the use of MPS and the distribution of *qacH*. Isolates harbouring *qacH* may be occasionally transferred to humans via animals or the community environment.

7.2.2 Relationship between QAC genes and disinfectant resistance

The use of disinfectants contributes to the emergence of QAC genes in staphylococci and resistance to a broad range of disinfectants (Smith, *et al.* 2008, Hegstad, *et al.* 2010). In this study, MICs and MBCs of BAK and CHX were compared between QAC genes positive and negative isolates.

Several *in vitro* studies have shown that long term exposure to sub-inhibitory concentrations of disinfectants could increase tolerance of strains to these agents and disinfectant resistance gene expression can be induced by exposure to sub-inhibitory concentrations of biocides (Smith, *et al.* 2008, Zhang, *et al.* 2011). Strains harbouring QAC genes were found to be more resistant to several disinfectants than gene negative strains (Smith, *et al.* 2008, Vali, *et al.* 2008, Furi, *et al.* 2013). Our results

demonstrated that MICs and MBCs of *Staphylococcus* carrying QAC genes to BAK and CHX were commonly higher than those of gene negative strains, which is consistent with previous studies. Some differences were observed between strains with different QAC genes. Isolates (two *S. aureus* and nine CNS) harbouring two or more QAC genes showed higher MICs and MBCs than other isolates having one gene only. Similar results have been found in previous studies, which may be due to additive effects of efflux pumps encoded by QAC genes (Zhang, *et al.* 2011).

The results of MIC and MBC of BAK and CHX to all isolates were less than 8mg/L, which was much lower than the actual used concentration of these disinfectants in clinical settings (McDonnell, *et al.* 1999, Rutala, *et al.* 2000). Strains carrying QAC genes could be destroyed by the application of disinfectants with high concentration and the increased resistance to disinfectants may have less clinical significance. However, it is worth noting that the efficacy of most disinfectants are unavoidably decreased due to dilution in actual application or presence of organic materials (Rutala, *et al.* 2004, Kawamura-Sato, *et al.* 2008, Stringfellow, *et al.* 2009). Of particular note is that commonly the concentrations of active agents in most MPS are not significantly higher than the MIC and MBC of these agents (Table 1.7). If not handled properly or being diluted, the disinfecting efficacy of MPS may decrease greatly and some strains, especially isolates carrying QAC genes, may survive and spread these genes among staphylococci species, which may explain the higher incidence of QAC genes in isolates from ortho-k lens wearers.

Therefore, in contact lens wearers, good compliance is essential as it can not only reduce the contamination rate of contact lenses and lens accessories, but also decrease the selective pressure for presence of strains carrying QAC genes.

7.2.3 Relationship between QAC genes and antibiotic resistance

Although the mechanisms of action between disinfectants and antibiotics are obviously different, their resistance mechanisms are very similar (Fraise 2002). Presence of QAC genes might not only reduce the effectiveness of disinfectants but also change bacterial susceptibilities to antibiotics (Sidhu, *et al.* 2001, Russell 2003). There is an increasing concern about possible links between the presence of QAC genes and antibiotic resistance.

Several previous studies demonstrated that resistance to several antibiotics (such as methicillin, gentamicin, macrolide, tetracycline, trimethoprim, and aminoglycosides) has frequently been reported in clinical isolates in association with QAC genes. Some antibiotic resistance genes can coexist with QAC genes on the same plasmids (Sidhu, *et al.* 2002, Russell 2003, Vali, *et al.* 2008, Zhang, *et al.* 2011, Zmantar, *et al.* 2011).

In this study, both *S. aureus* and CNS isolates carrying QAC genes (*qacA/B*, *smr* and *qacH*) were more resistant to tested antibiotics than those without QAC genes, which was consistent with previous reports (Smith, *et al.* 2008, Zhang, *et al.* 2011). The association with methicillin resistance was more obvious than other antibiotics,

significant differences being reached in both *S. aureus* and CNS. Genetic linkage between QAC genes and β -lactam antibiotic resistance genes, such as *blaZ*, has been reported in clinical and food industry isolates (Sidhu, *et al.* 2001, Sidhu, *et al.* 2002). Zhang *et al.* (2011) found that QAC genes were more commonly detected in MRSA strains. Significant higher resistant rates to several non- β -lactam antibiotics, including ciprofloxacin, tetracycline, imipenem and rifampicin, were detected in *S. aureus* harbouring QAC genes. Our results demonstrated that the presence of QAC genes may contribute to the cross-resistance between biocides and antibiotics. The strains harbouring QAC genes may have more chance to survive under treatment of these antibiotics with cross-resistance, which will increase the difficulty of treatment.

Genetic linkage between QAC genes and other antibiotic resistance genes has also been reported, such as *ermC*, *dfrA*, and *aacA-aphD* (Vali, *et al.* 2008, Zmantar, *et al.* 2011). However, the difference in the resistance to these antibiotics between strains carrying QAC genes or not did not reach significance and a larger sample size may be needed.

7.2.4 Efficacy of multipurpose solutions to *Staphylococcus* harbouring QAC genes

Currently, more than 90% of contact lens wearers use MPS to disinfect and clean their contact lenses and accessories, which plays a significant role in reducing the incidence of ocular infections (Lievens, *et al.* 2006). To secure FDA approval, before being released to the market, each solution shall be assessed on the antimicrobial efficacy

according to the stand-alone test and challenged with five microorganisms FDA recommended (Food-and-Drug-Administration 1997). However, the use of the limited panel of the stand-alone test may not represent the actual efficiency of MPS. Several studies demonstrated that presence of QAC genes in *Staphylococcus* contributed to reducing susceptibility to a broad of disinfectants, and some are main active agents of MPS (Food-and-Drug-Administration 1997, Smith, *et al.* 2008, Zhang, *et al.* 2011, Furi, *et al.* 2013). In this study, the efficacy of four commercially-available MPS for rigid lenses against *Staphylococcus* harbouring QAC genes were determined.

Consistent with previous observations, our results demonstrated that the presence of QAC genes in staphylococci reduced the disinfectant susceptibility, and in this case to MPS. Commonly, isolates harbouring QAC genes reached higher MIC and MBC and low log reduction to test MPS. However, not all reached significant difference between strains harbouring different QAC genes and gene negative strains, which may be due to small sample size.

The efficacy of almost all commercially-available FDA-approved MPS has been investigated in previous studies. They were all able to meet the requirement of 3-log reduction of viable bacterial cells for challenged organisms, including five FDA recommended strains and several non-FDA recommended clinical isolates (Leung, *et al.* 2004, Boost, *et al.* 2006, Boost, *et al.* 2010). Ideal results were also found in the test of two MPS against *Acinetobacter* carrying disinfectant resistant genes (Boost, *et al.* 2014). However, some unexpected results were detected in this study. Not all

isolates (including gene negative and positive strains) reached 3-log reduction under manufacturer's recommended conditions. Higher percentage of isolates harbouring two or more QAC genes failed to reach 3-log reduction than other strains, which was consistent with previous findings and may be due to additive effects of efflux pumps encoded by different QAC genes. In addition, some differences in strains with the same genes were observed in log reduction to each MPS. There may be some other factors contributing to reduced disinfecting efficacy of MPS which need to be further investigated.

Overall, the results demonstrated that long term used of MPS may contribute to higher carriage rate of *Staphylococcus* harbouring QAC genes in ortho-k lens wearers, which lead to reduced susceptibility to both biocides and antibiotics. MPS tested in this study were not effective enough to reach the FDA standard against some isolates and most of these strains were QAC gene positive. The results suggested that these strains may survive disinfection, which may increase the potential risks of ocular infection. Therefore, not only regular follow-up to examining the ocular health, but also close monitoring of the presence of isolates carrying QAC genes are recommended for each contact lens wearer, especially the children.

7.3 Limitations and scopes for further studies

This study successfully accomplished the objectives described in Chapter 2, but due to the limited time and resources, there are some limitations.

In this research, all subjects were children, aged from 7 to 14 years old, and recruited from the Optometry Clinic of The Hong Kong Polytechnic University. These subjects may be not representative of real distribution of QAC genes among contact lens wearers in Hong Kong. In this study, ortho-k lens wearers were required to wear their lenses every night and use the same MPS with 0.0005% PHMB. They were thought to have similar time of lens wear and exposure to MPS. However, this study is somewhat limited as the percentage of contact lens wearers using ortho-k lens worldwide is low (about 1%) and children only represent a small proportion of contact lens wearers (Morgan, *et al.* 2015). Most contact lens wearers are adults and use soft contact lenses. Thus, MPS for soft contact lenses are more commonly used and contain different active ingredients. Therefore, further work in this area is needed. Subjects may be divided into several groups according to age, types of contact lenses, types of MPS, and other factors and investigated for carriage of QAC positive staphylococci. The results may provide more useful information for the safe use of contact lenses.

To clearly describe the prevalence of QAC genes in a certain area, it is better to recruit subjects from several different sites to represent the whole target population. Mayer *et al.* (2001) analyzed the prevalence of *qacA/B* and *smr* in Europe based on the isolates collected from 24 hospitals in 14 countries. Zhang *et al.* (2011) recruited 455 nurses of 15 hospitals in Hong Kong to investigate the prevalence of several QAC genes in this population. Therefore, a multi-center study containing subjects with different ages is recommended.

This study determined the prevalence of QAC genes in staphylococci isolated from ortho-k lens and spectacle wearers, who were all healthily living in the community. Although ortho-k lens wearers are more likely to be exposed to disinfectants than the spectacle wearers, other information about subjects was not collected which may result in missing some risk factors contributing to acquiring QAC genes. Several studies have demonstrated that nurses or other medical workers may act as reservoirs to transfer pathogens from hospitals, nursing homes, long term care facilities to the general environments (Eveillard, *et al.* 2008, Zhang, *et al.* 2011, McDanel, *et al.* 2013). Some people may acquire pathogens due to frequently contacting with health care workers. In addition, due to their young age, the process of handling ortho-k lenses may be operated by subjects' parents, which may influence the results. Studies have shown that several disinfectant resistance genes were detected in isolates from some animals, such as dogs, cows, goats, and horses (Anthonisen, *et al.* 2002, Malik, *et al.* 2006). Locally, dogs are a popular pets kept by many families. If subjects had contacted with these animals, strains harbouring QAC genes may be transferred to them. As mentioned above, *qacG*, H, and J were seldom detected in human isolates, the source of isolates harbouring *qacH* found in this study needs to be further investigated. The relevant information should be evaluated, though this may bring remarkable difficulties due to large numbers of variables. Another limitation is that positive detection for QAC genes may be temporary or occasional, which may be due to unexpected contact and transferred from other people or the environment. Therefore, multiple detection for each subjects may be necessary for determine the carriage of strains harbouring QAC genes.

This study determined the carriage of *Staphylococcus* harbouring QAC genes in ortho-k lens and spectacle wearers, demonstrated that a higher prevalence of QAC genes were detected in staphylococci isolated from ortho-k lens wearers than spectacle wearers. However, not all reached significant differences. Only ten strains harbouring *qacH* and no strains harbouring *qacG* and *J* were found due to low prevalence and small sample size. This study also investigated the susceptibility of strains harbouring QAC genes to several antibiotics and two disinfectants and revealed that presence of QAC genes in staphylococci contributed to higher resistance rate to antibiotics and higher MIC and MBC to disinfectants. However, significant differences were not reached in all groups. The results demonstrated that isolates harbouring two or more QAC genes were more resistant to MPS, and similar results were found in previous studies. It may be due to additive effects of efflux pumps encoded by QAC genes, but only two *S. aureus* and nine CNS were challenged in this study. Therefore, a further large scale study including more subjects is needed to be performed to confirm the findings of this study.

The linkage between QAC genes and antibiotic resistance has been reported in several previous studies, but there are still many unknown questions about the association between QAC genes and increased resistance to antibiotics, which may lead to cross- and/or co- resistance to antibiotics (Russell 2003, Vali, *et al.* 2008). In this study, increased resistance to antibiotics was found in both *S. aureus* and CNS isolates carrying QAC genes. Genetic linkage between QAC genes and other antibiotic resistance genes has been reported in several studies, such as *blaZ* (β -lactam

antibiotics), *tetK* (tetracycline), *rpoB* (rifampicin), *ermC* (erythromycin), *dfrA* (trimethoprim), and *aacA-aphD* (gentamicin-kanamycin-tobramycin) (Sidhu, *et al.* 2001, Russell 2003, Vali, *et al.* 2008, Zmantar, *et al.* 2011). But our study did not provide clear evidence to explain increased resistance of QAC gene positive strains to antibiotics. In addition, phenotypic detection of antibiotic resistance may not reveal presence of genetic determination of antibiotic resistance. They may be silent and not expressed under certain conditions. These so-called silent antibiotic-resistance genes, have also been found in the genomes of *Staphylococcus* species, such as *mecA* (Wu, *et al.* 2001, Perreten, *et al.* 2005, Zhu, *et al.* 2007), *tetK* (Perreten, *et al.* 2005), *ermC* (Perreten, *et al.* 2005, Chancey, *et al.* 2012), *dfrA* (Perreten, *et al.* 2005,) and *blaZ* (Chancey, *et al.* 2012, Zhu, *et al.* 2007). These genes can be detected by PCR and their presence included in determining association between QAC genes and those for antibiotic resistance.

Several studies demonstrated that presence of QAC genes in staphylococci contributed to reduced susceptibility to a broad range of disinfectants, including CHX and PHMB (McDonnell, *et al.* 1999, Zhang, *et al.* 2011). Our results showed that the disinfecting efficacy of tested MPS decreased greatly due to the presence of QAC genes and some strains harbouring QAC genes did not reach 3-log reduction under recommended disinfecting conditions.

MPS used in this study are for rigid lenses and commonly have higher concentrations of active ingredients and/or stronger disinfecting efficacy than that of MPS for soft

contact lenses (Table 1.7). Strains harbouring QAC genes may have more chance to survive when disinfecting with MPS for soft contact lenses, which may lead to potential risks of ocular infection. In recent years, several new generation MPS have been developed and described as “dual disinfection” products because they each contain two significant chemical disinfectants. (Table 1.7) Further work is need to determine the disinfecting efficacy of these MPS for soft contact lenses, including new generation MPS, against strains harbouring QAC genes, which will provide more information for the safe use of contact lenses.

This study did reveal that ortho-k lens wearers had a higher risk of carrying staphylococci harbouring QAC genes than spectacle wearers and this risk was found to increase gradually from the commencement of ortho-k lens wear to 6 months. But this trend did not reach statistical significance. It is estimated that a minimum sample size of 30 subjects is required based on the results of Chapter 4 with 5% error. In addition, owing to the limited time and sample size, the longitudinal study did not go beyond the 6-month visit. There were some differences in the prevalence of QAC genes between 6-month and over one year ortho-k lens wearers. Extended studies are needed and beneficial to clearly characterize the emergence of QAC genes under selective pressure from use of MPS.

In summary, this is the first study to characterize the prevalence of QAC genes in staphylococci isolated from ortho-k lens wearers. This study determined that ortho-k lens wearers contributed to higher risk of presence of strains carrying QAC genes than

spectacle wearers and the prevalence of strains harbouring QAC genes increased gradually from the beginning of ortho-k lens wear to 6 months. Use of MPS may play a significant role in the emergence of strains harbouring QAC genes. Presence of QAC genes in staphylococci contributed to reduced susceptibility to antibiotics and disinfectants. A large proportion of strains harbouring QAC genes survived after being challenged with tested MPS under recommended disinfecting conditions. Surviving strains may colonize the periorbital region, contact lens and/or lens accessories for a long time and may cause ocular infections when the body immune system is reduced. The findings in this study provided deeper understanding of the bacteriological changes of ortho-k lens wearers and had significant clinical implication in safe use of contact lenses. Further studies in the area are needed due to the limitations and recommendations of this study.

APPENDIX

Appendix I Ethics Approval



To WONG Hie Hua (School of Optometry)

From LAM Kwok Cheung Andrew, Chair, Departmental Research Committee

Email sokclam@ Date 21-Mar-2012

Application for Ethical Review for Teaching/Research Involving Human Subjects

I write to inform you that approval has been given to your application for human subjects ethics review of the following project for a period from 01-Mar-2012 to 01-Sep-2014:

Project Title: Effects of long term use of contact lens disinfecting solutions on the presence of bacteria-harboring antiseptic-resistance genes in the conjunctival sac, eyelid and on the lens and lens accessories of orthokeratology lens wearers

Department: School of Optometry

Principal Investigator: WONG Hie Hua

Please note that you will be held responsible for the ethical approval granted for the project and the ethical conduct of the personnel involved in the project. In the case of the Co-PI, if any, has also obtained ethical approval for the project, the Co-PI will also assume the responsibility in respect of the ethical approval (in relation to the areas of expertise of respective Co-PI in accordance with the stipulations given by the approving authority).

You are responsible for informing the Departmental Research Committee in advance of any changes in the proposal or procedures which may affect the validity of this ethical approval.

You will receive separate email notification should you be required to obtain fresh approval.

LAM Kwok Cheung Andrew
Chair
Departmental Research Committee

Appendix II Consent Form (English Version)

Consent Form

Effects of long term use of contact lens disinfecting solutions on the presence of bacteria-harboring antiseptic-resistance genes in the conjunctival sac, eyelid and on the lens and lens accessories of orthokeratology lens wearers

- Have you read the information sheet provided? Yes / No
- Have you had an opportunity to ask questions and discuss this study? Yes / No
- Have your received satisfactory answers to all of your questions? Yes / No
- Have you received enough information about the study? Yes / No
- Who provided the information / answered your questions
Ms / Mr /Dr /Prof _____
- Do you understand that participation is entirely voluntary? Yes / No
- Do you understand that you are free to withdraw from the study
- at any time Yes / No
 - without having to give a reason Yes / No
 - without affecting your future care (*as applicable*) Yes / No
- Do you agree to take part in this study? Yes / No

Name (child):

Signature:

Date:

Name (parents or other guardian):

Signature:

Date:

Witness: (*as applicable*)

Appendix II Consent Form (Chinese Version)

知情同意書

長期應用隱形眼鏡藥水對角膜矯形鏡佩戴者眼部細菌耐藥性的影響

- 你已經閱讀了資訊調查表了嗎？ 是 / 否
- 你是否有機會提出關於這項研究的問題和參與討論？ 是 / 否
- 對於你的問題，你是否已經得到滿意的答案？ 是 / 否
- 關於這項研究，你是否已得到足夠的資料？ 是 / 否
- 誰為你提供的資訊和回答你的問題
- 石廣森先生 電話：2766 4470
- 曹黃惠華教授 電話：2766 6100
- 布慕蓮博士 電話：2766 8576
- 你知道參加這項研究是完全自願的嗎？ 是 / 否
- 你是否知道你可以在任何時候退出這項研究？ 是 / 否
- 你同意參加這項研究嗎？ 同意 / 不同意

兒童姓名：_____ 父母或者監護人姓名：_____

簽名：_____ 簽名：_____

日期：_____ 日期：_____

見證人：_____（如適用）

簽名：_____

日期：_____

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