Prevalence of Antiseptic-Resistance Genes in Staphylococci Isolated From Orthokeratology Lens and Spectacle Wearers in Hong Kong

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Purpose. To compare isolation of staphylococci from periorbital tissues and accessories of orthokeratology (ortho-k) lens and spectacle wearers and investigate prevalence of antiseptic-resistance (QAC) genes. To determine minimum inhibitory concentrations (MIC) of antiseptics and antibiotic susceptibility of isolates.

Methods. Staphylococci were isolated from eyelids, eyelashes, and conjunctival sacs of 23 ortho-k lens wearers and 20 spectacle wearers. Samples were also collected from ortho-k lenses, lens cases, and spectacle frames. Isolations of *Staphylococcus aureus* were compared between ortho-k subjects and controls for all samples and for coagulase-negative staphylococci (CNS) from conjunctival sacs. QAC genes were amplified in 110 *S. aureus* and 59 CNS isolates and prevalence compared in isolates from ortho-k lens and spectacle wearers. Associations were assessed between presence of QAC genes and antibiotic and antiseptic susceptibility.

Results. Although isolation of *S. aureus* did not differ significantly in periorbital samples from ortho-k and control subjects, QAC genes were significantly more common in both *S. aureus* and CNS from ortho-k subjects (odds ratio 4.4 and 10.74, respectively). Overall, *qac*A/B was the predominant gene detected, being present in 26.5% CNS and 11% *S. aureus*. *smr* and *qac*H were present in 12% of CNS, but were less common in *S. aureus*. QAC gene-positive isolates had higher MICs to benzalkonium chloride and chlorhexidine digluconate.

Conclusions. Our results suggest that long-term use of multipurpose solutions containing quaternary ammonium compounds may select for carriage of organisms harboring QAC genes. As these genes are associated with antibiotic resistance, their increased prevalence in isolates from contact lens wearers is a concern.

Keywords: staphylococci, antiseptic resistance genes, disinfectant, MIC, periorbital tissues, qac

Staphylococcus aureus and coagulase-negative staphylococci (CNS) can cause a wide range of diseases including soft tissue infection, keratitis, and endocarditis. Formerly, CNS was considered to be very rarely involved in disease, but since the 1980s the reported incidence of infections due to CNS has been increasing.[1,2]

To prevent the spread of pathogens and control infection, disinfectants based on quaternary ammonium compounds (QACs), such as benzalkonium chloride (BAK), and biguanides, such as chlorhexidine (CHG), have been extensively used in hospitals and other health care settings.[3,4] However, with the widespread use of these products, there is a growing concern about the emergence of disinfectant-resistant microorganisms[5] due to selective pressure in the environment, contributing to increasing difficulties in preventing infection.[6,7] 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.[8,9] There are two major groups of resistance genes, the major facilitator superfamily including *qac*A and *qac*B, and the small multidrug resistance family, which consists of *smr*, *qac*G, *qac*H, and *qac*J.[9,10]

One important application of disinfectant use is to ensure safe contact lens wear.[11] 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.[11,12] Most multipurpose solutions contain QACs and/or biguanides, which have a high level of activity against a wide range of common ocular pathogens.[13,14] 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 multipurpose solutions.

In this study we compared the prevalence of disinfectant-resistance genes harboring staphylococci colonizing the conjunctival sac and eyelid and on the lens and lens accessories of ortho-k lens wearers with those of spectacle wearers. In addition, the association between the presence of disinfectant-

resistance genes and antibiotic resistance determined in isolates was investigated.

Methods

Subjects

Twenty-three children (7–14 years) who were currently participating in an ortho-k project (myopic control study) at The Hong Kong Polytechnic University and who had been wearing ortho-k lenses for at least 1 year were recruited. A control group of 20 children wearing spectacles for myopic correction were also recruited from the same study. All subjects enjoyed good general health. Ocular health of all subjects was assessed by slit-lamp biomicroscopy. This study followed the tenets of the Declaration of Helsinki as revised in 2002, and ethics approval was obtained from the Departmental Research Committee of the School of Optometry.

Sample Collection

Samples were collected from the eyelids, the upper eyelashes, and the conjunctival sacs of all subjects, the contact lenses and the lens cases of ortho-k wearers, and the spectacle frames of the control group. For the conjunctival sac, a sterile swab soaked in sterile phosphate-buffered saline (PBS) was used to swab the lower conjunctiva of the left eye with a rolling motion from the lateral cantus toward the medial cantus. For the eyelid and eyelashes, two sterile cotton swabs moistened in sterile PBS were used to swab the central part of the lower eyelid and the eyelashes individually. The contact lens worn on the left eye was removed by the subject per normal routine and placed immediately in a new sterile lens case containing 2 mL sterile PBS, and the case was brought to the clinic for analysis. The subjects also brought their existing lens cases (empty) for microbial assessment. The lens case containing the lens was vortexed vigorously for 30 seconds to loosen any micro-organisms adhering to the lens surface. The lens was then removed from the case using sterile forceps and returned to the subject. The lens extract was poured into a bijou bottle containing 10 mL sterile brain heart infusion broth (BHI broth). For examination of flora from the routinely used lens case, a cotton swab soaked in Dey-Engley neutralizing broth (Oxoid, Basingstoke, UK) with 2 mM EDTA was used to sample the inner surfaces and the screw tops of the lens case; EDTA is used to help release bacteria from biofilms that may be present on the lens case surface. At this low concentration, EDTA is not bactericidal[15,16] and has been used in previous studies of contamination of lens cases.[17,18] For the spectacle frame, a sterile cotton swab moistened in sterile PBS was used to sample the right nose pad surface and the right arm of the spectacle frame. Each swab was broken off in a bijou bottle containing 10 mL sterile BHI broth and then vortexed for 30 seconds to release micro-organisms. After 16-hour incubation at 37°C, the broths were subcultured on SaSelect agar (Bio-Rad, Redmond, WA, USA) and incubated for 24 hours. For cultures showing growth, colonies displaying typical staphylococcal morphology were isolated, Gram stained, and identified using catalase, tube coagulase test, and Staphaurex Plus test (Murex Biotech Ltd, Dartford, UK). Strains positive for tube coagulase test and Staphaurex Plus test were reported as *S. aureus*, while those negative for these tests were reported as CNS. 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.

Antimicrobial Susceptibility

Antibiotic susceptibility profiles were determined by disc diffusion (Clinical Laboratory Standards Institute [CLSI] 2010) for the following agents: oxacillin, cefoxitin, erythromycin, chloramphenicol, clindamycin, gentamicin, ciprofloxacin, trimethoprim/sulfamethoxazole, tetracycline, penicillin G, imipenem, rifampicin, and fusidic acid.[19] Zones of inhibition were measured after 24-hour incubation at 37°C. *S. aureus* (25923; American Type Culture Collection, Bethesda, MD, USA) was used as a control strain. Interpretation of sensitivity and resistance was based on modified CLSI criteria.[19]

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

Minimum inhibitory concentrations and MBCs of BAK and CHG (Sigma-Aldrich Corp., St. Louis, MO, USA) were determined by the broth microdilution method (concentration range, 0.5–64 mg/L).[19] All QAC gene-positive isolates and 58 randomly selected negative isolates (30 *S. aureus* and 28 CNS) were tested.

DNA Isolation and *qac* Gene Detection

Plasmid DNA was extracted by a modified alkaline lysis method, with addition of lyostaphin and lysozyme (Sigma-Aldrich Corp.).[20] Polymerase chain reaction was performed for detection of *qac*A/B, *smr*, *qac*G, *qac*H, and *qac*J as previously described.[20] The amplified products were visualized by electrophoresis in 2% agarose and compared with those amplified from control *S. aureus* strains: TS77 (*qac*A/B), L20 (*smr*), RN4220 (pSK265, *qac*G), RN4220 (pSK265, *qac*H), and RN4220 (pSK265, *qac*J).

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 χ^2 or Fisher's exact test. Mann-Whitney *U* test was used to compare the MBC and MIC results.

Results

Table 1 shows a summary of isolates from study subjects. Ninety-three staphylococci (62 *S. aureus* and 31 CNS) were isolated from ortho-k subjects and 66 (48 *S. aureus* and 18 CNS) from the control subjects. *S. aureus* was present on the eyelids of 12 (52.2%), the eyelashes of 8 (34.8%), and the conjunctival sacs of 5 (21.7%) ortho-k subjects. *S. aureus* carriage rates on the eyelids (35%) and eyelashes (25%) of the control subjects were lower than those of ortho-k subjects, but these values did not reach statistical significance. The conjunctival sacs of eight (40%) control subjects were colonized with *S. aureus*. In comparison, colonization was observed in only five (21.7%) ortho-k subjects.

QAC genes were more common in isolates of CNS from the conjunctival sacs of ortho-k subjects (38.7%) than of control subjects (5.6%) ($P = 0.011$). Although the numbers of *S. aureus* isolates carrying QAC genes from the eyelids and eyelashes and conjunctival sacs were small, these genes were common in isolates from ortho-k subjects. Samples from lenses and lens cases yielded six and 13 *S. aureus* isolates, respectively, of which one from the lenses and two from the cases carried *qac*A/B. The lens isolate also carried *smr*. Spectacle frames yielded 14 *S. aureus* isolates, but none of these carried QAC genes (Table 1).

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Table 1. Origin of Staphylococcal Isolates

	S. aureus				Coagulase-Negative Staphylococci*			
		QAC Gene Positive (%)				QAC Gene Positive (%)		
Origin of Isolates	No. of Isolates	A/B	*smr*	H	No. of Isolates	A/B	*smr*	H
Orthokeratology group (% of subjects positive)								
Conjunctival sacs (22 *S. aureus*; 83 CNS)	5	1 (20)			31	12 (38.7)	4 (12.9)	4 (12.9)
Eyelid (52)	26	3 (11.5)	1 (3.8)					
Eyelash (35)	12	3 (25)	1 (8.3)	1 (8.3)				
Contact lens (22)	6	1 (16.7)	1 (16.7)					
Lens case (26)	13	2 (15.4)						
Total	62	10 (16.1)	3 (4.8)	1 (1.6)	31	12 (38.7)	4 (12.9)	4 (12.9)
Control group (% of subjects positive)								
Conjunctival sacs (40 *S. aureus*; 65 CNS)	10				18	1 (5.6)	2 (11.1)	2 (11.1)
Eyelid (35)	19	1 (5.3)	2 (10.5)					
Eyelash (25)	5	1 (20)						
Spectacle frames (35)	14							
Total	48	2 (4.2)	2 (4.2)		18	1 (5.6)	2 (11.1)	2 (11.1)

S. aureus: Ortho-k vs. control
Eyelid OR 2.03; 95% CI 0.144–1.689
Eyelash OR 1.6; 95% CI 0.166–2.356
Conjunctival sac OR 0.41; 95% CI 0.632–9.119
Presence of *qac*A/B OR 4.42; 95% CI 0.921–21.242 **($P = 0.046$)**
Presence of *smr* OR 1.17; 95% CI 0.188–7.292 ($P = 0.867$)

CNS: Ortho-k vs. control
Conjunctival sac OR 2.56; 95% CI 0.095–1.612
Presence of *qac*A/B OR 10.74; 95% CI 1.26–91.473 **($P = 0.011$)**
Presence of *smr* OR 1.19; 95% CI 0.195–7.217 ($P = 0.854$)
Presence of *qac*H OR 1.19; 95% CI 0.195–7.217 ($P = 0.854$)

P value in bold indicates significance. OR, odds ratio; CI, confidence interval.
* Isolates of CNS characterized only from conjunctival sacs.

QAC genes were more common in CNS (*qac*A/B 26.5%) than *S. aureus* (*qac*A/B 10.9%) ($P = 0.012$). Only one *S. aureus* and six CNS strains were positive for *qac*H. No sample was positive for *qac*G or *qac*J. Both *qac*A/B and *smr* were more frequently present in CNS and *S. aureus* from ortho-k than from control subjects, but the difference reached statistical significance only for *qac*A/B (CNS odds ratio = 10.74; confidence interval: 1.26–91.47; $P = 0.011$; *S. aureus* odds ratio = 4.42; confidence interval: 0.92–21.24; $P = 0.046$) (Table 1).

Resistance to most antibiotics was more likely in QAC gene-positive *S. aureus* isolates than in QAC gene-negative *S. aureus* isolates (Table 2). Isolates harboring QAC genes had higher median MICs (MIC_{50}) to BAK and CHG. The MBCs (both MBC_{50} and MBC_{90}) were also generally higher in QAC-positive strains, although not all reached significance due to small sample size (Table 3).

Discussion

In this study, QAC genes were found in staphylococci isolated from periorbital tissues and accessories of ortho-k and control subjects. Carriage rates of *S. aureus* in children in the periorbital region, especially in the conjunctival sac, were higher than previously reported (range, 6%–16.4%).[21,22] 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. 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 multipurpose solutions that could kill or limit the growth of flora in the conjunctival sac. In contrast, carriage rates of *S. aureus* were higher on the eyelids and eyelashes of ortho-k subjects than those of spectacle controls. 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 both on the lenses and in the lens cases of ortho-k subjects. Contamination of the lens (>25%) was a combination of organisms from the eye and 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 between these procedures. Removal of lenses involved touching the periorbital tissues with the fingers before handling the left lens. At least one CFU of *S. aureus* was present in 22% of lens cases. This fits well with findings of a recent study reporting that only 0% to 9% of lens cases yielded at least 20 CFU *S. aureus*.[23]

Isolations 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 that have been in contact with nasal pharyngeal secretions, the anterior nares being the primary site of colonization for *S. aureus*.[24] However, two of the isolates from lens cases carried *qac*A/B, while none of those from spectacle frames were positive for antiseptic-resistance genes. 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.

The presence of QAC genes in staphylococci has been reported in a number of studies performed in Hong Kong,[20] Japan,[25] Europe,[26] and North America.[27,28] However, this is the first study to investigate their presence in the normal flora of the eye and contact lenses and accessories. It was found that *qac*A/B and *smr* were widely distributed among the staphylococci isolates from ortho-k lens and control subjects, but only one *S. aureus* isolate and six CNS isolates harbored *qac*H. The higher incidence of *qac*A/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. There was no significant difference in the presence of other QAC genes between the two groups. Prevalence of both *qac*A/B and *smr* was significantly higher

Table 2. Antimicrobial Susceptibility Patterns of *S. aureus* and CNS Isolates

	S. aureus					CNS				
Antibiotics	**All, *n* = 110 R (%)**	**QAC, +ve, *n* = 15 R (%)**	**QAC, -ve, *n* = 95 R (%)**	**OR**	***P***	**All, *n* = 49 R (%)**	**QAC, +ve, *n* = 20 R (%)**	**QAC, -ve, *n* = 29 R (%)**	**OR**	***P***
Oxacillin	N/A	N/A	N/A			14 (28.6)	9 (45.0)	5 (17.2)	3.93	**0.035**
Cefoxitin	3 (2.7)	2 (13.3)	1 (1.1)	14.46	**0.007**	NA	NA	NA		
Erythromycin	51 (46.4)	8 (53.3)	43 (45.3)	1.38	0.560	14 (28.6)	8 (40.0)	6 (20.7)	2.56	0.141
Chloramphenicol	8 (7.3)	2 (13.3)	6 (6.3)	2.28	0.331	4 (8.2)	3 (15.0)	1 (3.4)	4.94	0.147
Clindamycin	11 (10.0)	3 (20.0)	8 (8.4)	2.72	0.165	10 (20.4)	5 (25.0)	5 (17.2)	1.6	0.508
Gentamicin	6 (5.5)	2 (13.3)	4 (4.2)	3.5	0.148	4 (8.2)	3 (15.0)	1 (3.4)	4.94	0.147
Ciprofloxacin	2 (1.8)	2 (13.3)	0 (0.0)			7 (14.3)	3 (15.0)	4 (13.8)	1.1	0.906
Trimethoprim/ sulfamethoxazole	2 (1.8)	1 (6.7)	1 (1.1)	6.71	0.130	1 (2.0)	1 (5.0)	0 (0.0)		
Tetracycline	17 (15.5)	6 (40.0)	11 (11.6)	5.09	**0.005**	10 (20.4)	5 (25.0)	5 (17.2)	1.6	0.508
Penicillin G	87 (79.1)	13 (86.7)	74 (77.9)	1.84	0.438	28 (57.1)	13 (65.0)	15 (51.7)	1.98	0.356
Imipenem	1 (0.9)	1 (6.7)	0 (0.0)			2 (4.1)	1 (5.0)	1 (3.4)	1.47	0.627
Rifampicin	3 (2.7)	2 (13.3)	1 (1.1)	14.46	**0.007**	4 (8.2)	2 (10.0)	2 (6.9)	1.5	0.481
Fusidic acid	3 (2.7)	1 (6.7)	2 (2.1)	3.32	0.313	6 (12.2)	3 (15.0)	3 (10.3)	1.53	0.376

P value in bold indicates significance. R, resistant; N/A, not applicable; +ve, positive; -ve, negative.

in CNS than *S. aureus* as was similarly reported in several recent studies.[20,29,30]

Most disinfectant-resistance genes are plasmid borne and can spread between staphylococcal species.[31] Coagulase-negative staphylococci, the most common organism in normal flora, may have a higher potential to harbor these genes. Our results showed that MICs and MBCs of *S. aureus* and CNS with QAC genes to BAK and CHG were higher than those of gene-

Table 3. MICs and MBCs of Benzalkonium Chloride and Chlorhexidine Digluconate for *S. aureus* and CNS With and Without QAC Genes

			MIC, mg/L				MBC, mg/L			
Strains	**QAC Genes**	**No.**	**Range**	**MIC_{50}**	**MIC_{90}**	***P***	**Range**	**MBC_{50}**	**MBC_{90}**	***P***
Benzalkonium chloride										
S. aureus	-	30	0.5–4	1	4		1–8	2	4	
	A/B	10	0.5–4	2	4	**0.011**	4–8	4	8	**0.011**
	smr	2	0.5–4	2	4	0.516	4–8	4	8	0.076
	A/B+*smr*	1	4			N/A	4			N/A
	A/B+*smr*+H	1	4			N/A	4			N/A
	qac positive	14	0.5–4	2	4	**0.004**	4–8	4	8	**0.003**
CNS	-	28	0.5–2	1	2		0.5–4	2	4	
	A/B	8	0.5–4	2	4	0.126	2–8	2	8	0.689
	smr	4	0.5–4	2	4	0.270	2–8	4	8	0.184
	A/B+*smr*	2	2	2	2	0.174	4	4	4	0.221
	H	3	2–4	4	4	**0.011**	4–8	4	8	**0.044**
	A/B+H	3	2–4	2	4	**0.036**	4–8	4	8	**0.044**
	A/B+*smr*+H	1	2			N/A	4			N/A
	qac positive	21	0.5–4	2	4	**0.002**	1–8	4	8	**0.024**
Chlorhexidine digluconate										
S. aureus	-	30	0.5–2	0.5	1		0.5–4	1	2	
	A/B	10	0.5–2	1	2	**0.029**	1–8	2	4	**0.012**
	smr	2	0.5–1	0.5	1	0.840	1–2	1	2	0.546
	A/B+*smr*	1	1			N/A	2			N/A
	A/B+*smr*+H	1	1			N/A	2			N/A
	qac positive	14	0.5–2	1	2	**0.020**	1–8	2	4	**0.007**
CNS	-	28	0.5–1	0.5	1		0.5–2	1	2	
	A/B	8	0.5–2	1	2	**0.012**	1–4	2	4	**0.006**
	smr	4	0.5–2	0.5	2	0.259	1–4	1	4	0.071
	A/B+*smr*	2	1	1	1	**0.042**	2–4	2	4	**0.007**
	H	3	0.5–1	1	1	0.187	1–2	2	2	**0.049**
	A/B+H	3	0.5–2	1	2	0.101	1–4	1	4	0.258
	A/B+*smr*+H	1	1			N/A	2			N/A
	qac positive	21	0.5–2	1	2	**0.002**	1–4	2	4	<0.001

P value in bold indicates significance.

negative strains, although there were some differences between strains with different QAC genes. The MICs and MBCs of the two antiseptics tested for all isolates were lower than actual concentrations recommended for disinfection.[20] However, disinfectants in real-life situations for lens and lens accessories are inevitably diluted by water, saline, and tears, which will decrease the effects of the disinfectants. In addition, effectiveness of multipurpose solutions can decrease during storage of the opened solutions.[32] Reduction in effectiveness can allow more strains harboring 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 subinhibitory concentrations of biocides.[33] Strains harboring QAC genes may be more likely to survive the disinfection process and serve as a source of infection.

Previous investigators have also reported antibiotic resistance of isolates as closely associated with the presence of QAC genes.[20,27,34] Gentamicin, macrolide, and other antibiotic-resistance genes can coexist with QAC genes on the same plasmids.[7,30] Resistance to several antibiotics (tetracycline, trimethoprim, and aminoglycosides) has frequently been reported in clinical isolates in association with QAC genes.[20,30,35,36] Our results showed that resistance to cefoxitin, tetracycline, and rifampicin in *S. aureus* and to oxacillin in CNS was significantly higher in isolates harboring QAC genes. The association between QAC genes and antibiotics may increase the risk of antibiotic-resistant infection. As noncompliance with correct routines of contact lens wear is the leading risk factor for microbial keratitis, the increased potential for these infections to be caused by more resistant organisms that may be more difficult to treat is of concern.

In summary, long-term use of antiseptics may contribute to a higher presence of QAC genes among staphylococci species isolated from ortho-k lens wearers. This may increase the risk of infection with an antibiotic-resistant organism. It is important that disinfection of lenses be performed correctly, avoiding dilution of disinfection solution by topping up or use of expired solutions that may have reduced disinfecting power, enhancing the survival of organisms carrying antiseptic-resistance genes.

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