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Benzalkonium Chloride Resistance in *Staphylococcus epidermidis* on the Ocular Surface of Glaucoma Patients Under Long-Term Administration of Eye Drops

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Purpose: We previously reported the presence of multidrug-resistant staphylococci on the ocular surface of glaucoma patients using prostaglandin analog drops for more than 1 year. Here, we investigated the effect of benzalkonium chloride (BAC) on these multidrug-resistant staphylococci.

Methods: *Staphylococcus epidermidis* was isolated from the conjunctival sacs of 32 eyes of 32 patients comprised of 13 eyes treated with 0.005% latanoprost (Xalatan; Xa group) and 19 eyes treated with 0.004% travoprost (Travatan Z; Tz group). The minimum inhibitory concentrations (MICs) of prostaglandin analogs and BAC were measured. The presence of efflux pump genes was analyzed using polymerase chain reaction.

Results: No difference was found in the MIC values of prostaglandin analogs. In contrast, the MIC values of BAC were significantly higher for the isolates from the Xa group than for those from the Tz group (2.02 vs. 1.02 μg/mL; $P = 0.001$). One proton-motive efflux gene, *qacC/smr*, was detected more frequently in the Xa isolates than in the Tz isolates ($P < 0.001$). The prevalence of methicillin resistance was correlated with the presence of *qacC/smr* ($P = 0.010$), and the MIC of BAC was significantly correlated with the detection of *qacA/B* and *qacC/smr* sequences ($P = 0.03$ and $P < 0.001$, respectively).

Conclusions: The long-term use of eye drops containing BAC might select BAC-resistant *S. epidermidis* harboring *qacC/smr*.

Translational Relevance: These findings suggest that the long-term use of eye drops containing BAC might be inappropriate in terms of avoiding antimicrobial resistance.

Introduction

Quaternary ammonium cations (QACs) are common biocides and disinfectants that have been used for 50 to 100 years.[1,2] QACs are cationic detergents that lower the surface tension to arrest the growth of or kill bacteria. Among QACs, benzalkonium chloride (BAC) is routinely used in ophthalmic solutions as a preservative.

In response to disinfectants, bacteria have developed mechanisms to effectively resist them.[3] Proton motive force efflux pumps, which can expel intracellular drugs, are one of the major mechanisms through which bacteria acquire such resistance. Several bacteria have their own efflux pumps; in *Staphylococcus*, *qacA*,[4] *qacB*,[5] and *qacC*/*smr*[6,7] genes encoding efflux pumps have been detected. The *qacA*/*B* genes contain 1542 nucleotides and encode proteins of 514 amino acid residues with 14 transmembrane

Table 1. Eye Drops Used in This Study

	Xalatan (0.005%)	Travatan Z Ophthalmic Solution (0.004%)
Main agent	Latanoprost	Travoprost
Preservative	Benzalkonium chloride	Boric acid, zinc chloride
Company	Pfizer, Inc.	Alcon Japan

segments. QacA and QacB differ from each other at amino acid residues 291, 323, and 380. QacA is responsible for resistance to monovalent and divalent organic cations, whereas QacB provides resistance to only monovalent organic cations.[5,8] The *qacA*/*B* genes localize to mobile DNA elements such as plasmids, transposons, and integrons.[9] The *qacC*/*smr* genes, which are 342 nucleotides in length, encode QacC/SMR, a 107-amino-acid protein with four transmembrane segments.[7,8] The *qacC*/*smr* genes are known to localize to plasmids of less than 3 kb.[9] Recently, staphylococci with *qac* genes have been widely detected in both humans and animals, and this could have influenced the outbreak of multidrug-resistant bacteria.[9]

Glaucoma patients are routinely prescribed eye drops to reduce their intraocular pressure as a long-term treatment. Our previous study showed that methicillin-resistant *Staphylococcus epidermidis* (MRSE) was isolated more frequently in patients treated with 0.005% latanoprost (Xalatan [Xa]; Pfizer, Inc., New York, NY), which contains BAC, than in patients treated with 0.004% travoprost (Travatan Z [Tz]; Alcon Japan Co. Ltd., Tokyo, Japan), which does not contain BAC.[10] In the current study, we aimed to clarify the factors related to the methicillin susceptibility of *S. epidermidis* in the conjunctival flora. Specifically, we analyzed the susceptibility of *S. epidermidis* isolated in our previous report[10] to main prostaglandin agents and BAC and examined whether the isolated *S. epidermidis* contained the *qacA*/*B* and *qacC*/*smr* genes.

Methods

Staphylococci Isolates

Thirty-two strains of *S. epidermidis* isolated in our previous study were analyzed. Nineteen strains were isolated from 19 patients using Xa (Xa group), and 13 strains were isolated from 13 patients using Tz (Tz group). Table 1 shows the main agents, preservatives, and companies associated with each eye drop. After these strains were isolated and identified, they were preserved at –80°C in a Microbank vial (Pro Lab Diagnostics, Tokyo Japan). The study design was approved by the ethical review board of Miyata Eye Hospital, and all of the experiments were conducted in accordance with the tenets of the Declaration of Helsinki and the ethical guidelines for clinical research. The research study was registered in the UMIN Clinical Trials Registry (UMIN000019650). All subjects provided informed consent, and written permission was obtained.

One bead from each Microbank vial was spread on Muller–Hinton (MH) agar and incubated overnight at 37°C. The cell cultures were prepared via the inoculation of one colony in 5 mL MH broth, which was incubated at 37°C with shaking. Isolated strains were identified with the MALDI-TOF MS Biotyper (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions.

Susceptibility of Isolates to Prostaglandins and BAC

The minimum inhibitory concentrations (MICs) of the prostaglandins latanoprost and travoprost (Cayman Chemical, Ann Arbor, MI), as well as BAC (Kanto Chemical Co., Inc., Tokyo, Japan), against *S. epidermidis* were measured by the broth microdilution method.

Preparation of Bacterial DNA

Bacterial colonies cultured overnight on MH agar were suspended in 0.2 mL of Tris-ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0). After boiling for 5 minutes, bacterial DNA was extracted using the phenol/ chloroform method, precipitated in ethanol, and resuspended in distilled water. The extracted DNA samples were used as a template for polymerase chain reaction (PCR) analysis.

PCR Amplification of *qac* Genes

The presence of *qacA*/*B* and *qacC*/*smr* sequences was analyzed using the following two sets of primers: 5′-GCA GAA AGT GCA GAG TTC G-3′ and 5′-CCA GTC CAA TCA TGC CTG-3′ for *qacA*/*B* (amplified product size 361 bp)[4] and 5′-GCC ATA AGT ACT GAA GTT ATT GGA-3′ and 5′-GAC TAC GGT TGT

Table 2. Minimum Inhibitory Concentration of Various Prostaglandin Eye Drops (Latanoprost, Travoprost, and BAC) Against *S. epidermidis* Isolates from the Ocular Surface of Glaucoma Patients

	MIC (μg/mL)		
Isolates	Latanoprost	Travoprost	BAC, Mean ± SD
Isolates from Xa group			
Total ($n = 19$)	>100	>80	2.02 ± 1.86[a]
MSSE ($n = 3$)	>100	>80	0.98 ± 2.89
MRSE ($n = 16$)	>100	>80	2.31 ± 1.55
Isolates from Tz group			
Total ($n = 13$)	>100	>80	1.02 ± 1.57[a]
MSSE ($n = 10$)	>100	>80	1.03 ± 1.62
MRSE ($n = 3$)	>100	>80	0.98 ± 1.55

MSSE, methicillin-sensitive *S. epidermidis*; MRSE, methicillin-resistant *S. epidermidis*.
[a]Significant differences in total and MRSE isolates from Xa and Tz groups based on a *t*-test ($P = 0.001$).

TAA GAC TAA ACC T-3′ for *qacC/smr* (amplified product size 195 bp).[6] The cycle conditions were as follows: an initial DNA denaturation step of 96°C for 3 minutes, 25 cycles of 94°C for 20 seconds, 53°C for 20 seconds, and 72°C for 20 seconds, and a final extension step at 72°C for 5 minutes.[11] PCR products were analyzed by 2% agarose gel electrophoresis. All results were confirmed by at least two independent experiments.

Sequencing Analysis

After purification using a PCR purification kit (Qiagen, Hilden, Germany) or ExoSAP-IT PCR product cleanup reagent (Thermo Fisher Scientific, Waltham, MA), the PCR products were sequenced on both strands by the Sanger method using the same primers used for PCR.

Statistical Analysis

The MIC values of prostaglandin analogs and BAC were compared between the Xa and Tz groups using *t*-tests. The correlation between the presence of the *qacA/B* and *qacC/smr* sequences and whether or not eye drops were used was examined using Fisher's exact test. In addition, the correlation between the detection of the *qacA/B* and *qacC/smr* sequences and methicillin sensitivity was also examined using Fisher's exact test. Linear regression analysis was used to assess the association between the MIC of BAC and the presence of *qacA/B* and *qacC/smr*.

Results

Determination of the Component of Eye Drops That Inhibits Bacterial Growth

To identify which component of the eye drops inhibited the growth of *S. epidermidis* isolates, we determined the MIC of pure latanoprost, pure travoprost, and BAC against the isolates and found no inhibition of the growth of the isolates by either of the prostaglandins at concentrations of 80 or 100 μg/mL. However, BAC exerted an inhibitory effect on the isolates (Table 2). In addition, the mean MIC of BAC was higher for methicillin-resistant isolates of the Xa group than for methicillin-resistant isolates of the Tz group ($P = 0.001$). Meanwhile, no significant difference between the two groups was found with respect to methicillin-sensitive *Staphylococcus* isolates.

PCR and Sequencing Analysis of *qacA/B* and *qacC/smr*

Using the established PCR method to detect efflux pumps of staphylococci, we examined the presence of *qacA/B* and *qacC/smr* sequences in the isolates from the two groups (Table 3). No statistically significant difference was observed between Xa and Tz groups based on detection of the *qacA/B* sequence, whereas the *qacC/smr* sequence was detected at significantly different rates between the isolates from the two groups. In addition, we found that the *qacC/smr* sequence was more frequently detected in methicillin-resistant *Staphylococcus* isolates than in methicillin-sensitive isolates (Table 4).

Table 3. Presence of *qacA/B* and *qacC/smr* Gene Sequences in *S. epidermidis* Isolates from Groups Using Different Eye Drops

Gene Sequence	Isolates from Xa Group ($n = 19$)	Isolates from Tz Group ($n = 13$)	*P*
qacA/B			1.00
+	7	4	
–	12	9	
qacC/smr			<0.001
+	15	2	
–	4	11	

Statistical analysis using Fisher's exact test.

Table 4. Presence of *qacA/B* and *qacC/smr* Gene Sequences in *S. epidermidis* Isolates with Respect to Methicillin Sensitivity

	Number of Isolates		
Gene Sequence	Methicillin-Resistant	Methicillin-Sensitive	*P*
qacA/B			1.00
+	7	4	
–	12	9	
qacC/smr			0.01
+	14	3	
–	5	10	

Statistical analysis using Fisher's exact test.

Table 5. Clonality of *qacA/B* Sequences of *S. epidermidis* Isolates Amplified by PCR

Sequence	Change in Nucleotide and Amino Acid Residue	BLAST Search (100% Match): Plasmid Name (GenBank)	Clone No.
Type 1	Prototype	pSK156 (61835)	2
Type 2	c.542 G>C p.V181L, c.651 T>C p.F217S	pHOB1 (CP018843)	3
Type 3	c.555 T>C p.V181L, c.651 T>C p.F217S	pKG-18 (AP019544.1)	6

After sequencing the amplified PCR products of the isolates, basic local alignment search tool analysis (BLAST) was performed. The sequences of amplified *qacA/B* and *qacC/smr* products were found to have 100% identities with the *qacA/B* and *qacC/smr* sequences, respectively, deposited in the GenBank database. The sequences of *qacC/smr*-amplified products were homogeneous without base changes, whereas the sequences of *qacA/B* were heterogeneous with three variants in some amino acid residues (Table 5; Supplementary Fig. S1). No specific linkage was observed for the variants with respect to isolates from Xa or Tz groups (data not shown).

Correlation Between MIC of BAC and the Presence of *qacA/B* and *qacC/smr*

Finally, we analyzed the correlation between the MIC of BAC against the isolates and the presence of *qacA/B* and *qacC/smr*. For *qacA/B*, MIC values tended to be higher when the isolates contained these genes. Similarly, for the isolates positive for the *qacC/smr* sequence, the MIC of BAC was significantly higher (Fig.). In addition, the *qacA/B* and/or *qacC/smr*-positive *Staphylococcus* isolates in the Xa group tended to be associated with high MICs for various antibiotics such as carbapenem, fluoroquinolone, or erythromycin (Table 6).

Table 6. Correlation Between PCR Detection of *qacA/B* and *qacC/smr* Gene Sequences and Susceptibility Patterns of *S. epidermidis* Isolates

	PCR		MIC (μg/mL)							
	qacA/B	*qacC/smr*	BAC	MPIPC	CAZ	IPM	MFLX	EM	VCM	LZD
Xa Group										
1	−	−	0.39	≤0.25	2	≤0.063	≤0.063	0.25	1	1
2	−	−	0.78	≤0.25	2	≤0.063	0.125	0.25	1	1
3	−	+	0.78	2	8	0.125	1	>128	2	1
4	−	+	1.56	1	8	≤0.063	2	>128	2	1
5	−	+	1.56	≤0.25	1	≤0.063	≤0.063	4	1	1
6	−	+	1.56	≤0.25	4	≤0.063	0.125	64	2	2
7	−	+	1.56	>8	16	0.5	64	>128	2	1
8	+	−	1.56	>8	32	0.5	1	0.25	2	1
9	+	−	3.13	4	16	0.5	1	0.25	1	1
10	−	+	3.13	>8	16	1	32	>128	2	1
11	−	+	3.13	4	8	0.125	0.125	64	2	1
12	+	+	3.13	>8	32	4	1	32	2	1
13	−	+	3.13	>8	32	4	1	>128	2	1
14	+	+	3.13	≤0.25	2	≤0.063	1	32	2	0.5
15	−	+	3.13	>8	32	1	2	0.5	2	1
16	+	+	3.13	>8	32	2	1	64	2	1
17	+	+	3.13	>8	32	1	2	64	2	1
18	−	+	3.13	>8	32	2	1	0.25	2	1
19	+	+	3.13	>8	16	0.25	1	64	2	1
Total	7	15								
Tz Group										
20	−	−	0.39	≤0.25	4	≤0.063	0.125	0.25	2	0.5
21	−	−	0.78	≤0.25	4	≤0.063	0.125	0.25	2	1
22	−	−	0.78	≤0.25	4	≤0.063	0.25	0.25	1	1
23	+	−	0.78	≤0.25	2	≤0.063	0.125	0.25	2	1
24	−	−	0.78	≤0.25	2	≤0.063	0.125	0.25	2	1
25	−	−	0.78	>8	16	0.125	0.125	>128	2	1
26	−	−	0.78	2	8	0.125	0.125	16	2	1
27	−	+	1.56	≤0.25	4	≤0.063	0.125	0.25	2	1
28	−	−	1.56	≤0.25	4	≤0.063	0.125	0.25	2	2
29	+	−	1.56	≤0.25	8	≤0.063	1	32	2	1
30	+	−	1.56	>8	32	0.5	2	0.25	2	1
31	−	+	1.56	2	8	0.125	1	0.25	1	1
32	+	−	1.56	≤0.25	4	≤0.063	≤0.063	32	2	1
Total	4	2								

Numbers with shaded background are determined to be resistant or intermediate according to the Clinical & Laboratory Standard Institute. MPIPC, methyl-phenyl-isoxazolyl penicillin; CAZ, ceftazidime; IPM, imipenem; MFLX, moxifloxacin; EM, erythromycin; VCM, vancomycin; LZD, linezolid.

Discussion

In a previous study, we reported that methicillin-resistant *S. epidermidis* occurred more frequently on the ocular surface of glaucoma patients subjected to the long-term use of Xa compared to that for subjects treated with Tz.[10] These isolates were obtained from the indigenous flora of the conjunctiva of glaucoma patients treated with prostaglandin analog monotherapy for at least 1 year. In the current study, we analyzed these *S. epidermidis* isolate stocks and found that

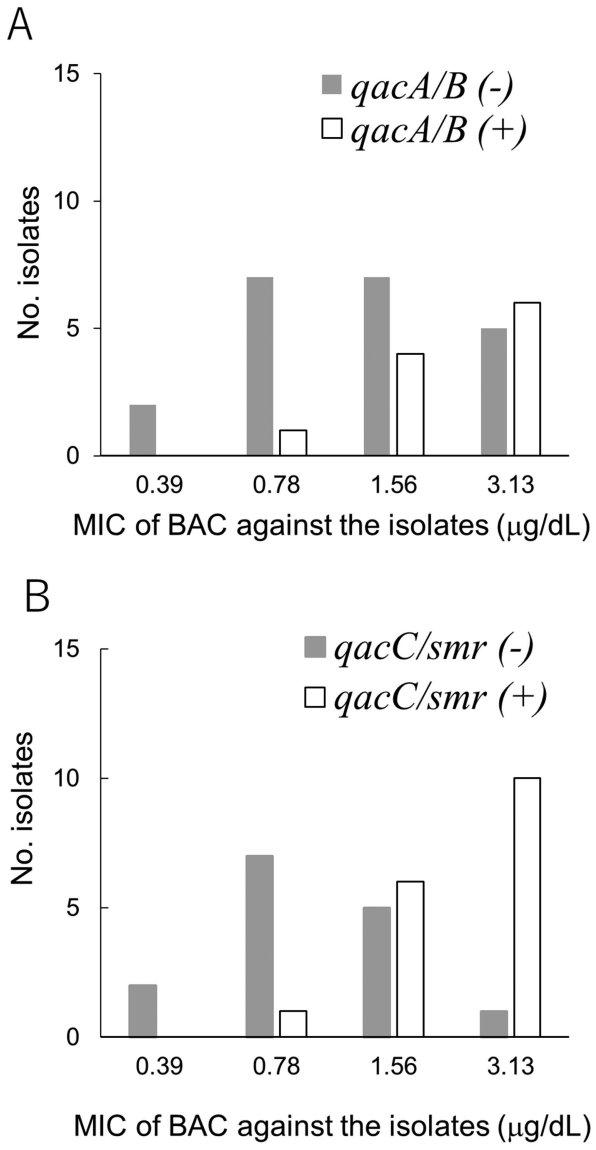

Figure. Correlation between the efflux pump genes detected by PCR methods and the MIC of BAC against *S. epidermidis* isolates. (A) The number of isolates positive (*open bar*) or negative (*closed bar*) for the *qacA/B* gene. (B) The number of isolates positive (*open bar*) or negative (*closed bar*) for the *qacC/SMR* gene. There was a significant correlation between the MIC of BAC and the detection of *qacA/B* and *qacC/smr* sequences ($P = 0.03$ and $P < 0.001$, respectively).

those obtained from the ocular surface of glaucoma patients under Xa treatment exhibited resistance to BAC as indicated by higher MIC values (Table 2), and, especially, methicillin-resistant isolates were significantly resistant to BAC. This higher MIC of BAC against *S. epidermidis* isolates was also correlated with detection of the *qacC/smr* sequence.

We initially focused on identifying the component of eye drops that plays the key role in the appearance of BAC-resistant *Staphylococcus* on the ocular surface. Although arachidonic acid, which is a precursor of prostaglandins, is known to inhibit bacterial proliferation,[12] no antibiotic effect of prostaglandins was observed based on in vitro analysis in this study. In contrast, BAC showed a prominent inhibitory effect on *Staphylococcus*, as expected.

The high MIC of BAC observed for *S. epidermidis* isolates from the patients treated with Xa suggested that BAC-resistant bacteria were selected on the ocular surface exposed to BAC-containing ocular drops. The mechanism of this resistance has been investigated by several groups.[5–9] It has been demonstrated that the bacterial acquisition of efflux pump systems localized in the bacterial cell membrane play an important role in the efflux of BAC from the bacterial cytoplasm and reduce intracellular BAC concentrations.[13]

Several efflux pumps, such as those encoded by *qacA/B* and *qacC/smr* genes, have been identified in *Staphylococcus*.[13] From the results of this study, it became apparent that acquisition of the *qacC/smr* gene was significantly correlated with the presence of BAC-resistant *Staphylococcus* on the ocular surface after prolonged exposure to BAC-containing eye drops. Thus, the product of *qacC/smr* on the bacterial membrane could result in the efflux of BAC from the bacterial cytoplasm.[14]

Shi et al.[15] reported that staphylococci with *qac* genes could be found on the ocular surface with long-term orthokeratology lens use. Because contact lens solutions generally contain some disinfectants, orthokeratology lens users are likely constantly exposed to BAC/QAC. The results of this study differed from ours; in their study, the positive ratio of *qacA/B* genes in the isolates was higher than that of *qacC/smr* genes among users of the contact lens. In contrast, our results showed that the resistance to BAC seen in the isolates from the Xa group could be correlated with the increased detection of *qacC/smr* genes. Although the difference between *qacA/B* or *qacC/smr* genes of *S. epidermidis* has not been sufficiently addressed in the literature, this study showed that the long-term use of BAC-containing eye drops increases the prevalence of *S. epidermidis* harboring *qacC/smr* on the ocular surface.

We speculated that the *qacC/smr*-positive staphylococci had selectively survived on the ocular surface exposed to eye drops containing BAC/QAC. These staphylococci might have acquired *qacC/smr* genes through transduction, bacterial conjugation, and transformation.[16] The mechanisms through which antibiotic resistance is acquired have been elucidated previously.[16–18] Alterations to the penicillin-binding protein 2A, which is encoded by *mecA*, decrease binding affinity to methicillin and confer a methicillin-resistant property to the bacteria.[19,20] In

methicillin-resistant staphylococci, the *mecA* gene and other antibiotic resistance genes are present together and are called SCC*mec*.[21] SCC*mec* causes staphylococci to be multidrug resistant. Although *qacA*/*B* and *blaZ*, a β-lactamase gene, are known to co-localize on the same mobile DNA element,[22,23] it has not been demonstrated whether or not *mecA* and *qacC*/*smr* genes are co-localized on the same mobile DNA element. Therefore, we hypothesize that *Staphylococcus* isolates independently bearing *mecA* and *qacC*/*smr* genes tended to survive in the environment exposed to eye drops containing BAC.

Some methicillin-resistant *Staphylococcus aureus* (MRSA) strains resist BAC more than methicillin-sensitive *S. aureus*.[24] MRSA cultured in a BAC-rich environment for 72 hours in a dish becomes more resistant not only to BAC but also to ofloxacin.[25] These results indicate that exposure to BAC might select BAC-resistant *Staphylococcus*, probably through the induction of efflux pump proteins. Thus, the MIC of antibiotics could increase after the induction of efflux pump proteins. Although the number of isolates of staphylococci examined in this study was rather small, the selection and induction of efflux pumps in bacteria were likely to occur in the indigenous flora on the ocular surface following prolonged exposure to eye drops containing BAC. Further studies are required to confirm this phenomenon.

We examined only the *qacA*/*B* and *qacC*/*smr* genes of the *Staphylococcus* isolates using PCR and their sequences using the Sanger method in this study. If we were to analyze other bacteria isolated from the ocular surface exposed to BAC-containing eye drops by using next-generation sequencing techniques,[26] we might find similar changes in the bacterial and plasmid genes. This possibility must be clarified in the future.

To preserve eye drops, it is very important to maintain the bottles under sterile conditions during their use for topical administration by patients. Although BAC has been one of the most reliable preservatives and has been in use for more than 50 years, the results of this study indicate that glaucoma patients treated with eye drops containing BAC are at risk of possessing BAC-resistant staphylococci on their ocular surface. Moreover, these staphylococci are possibly resistant to other antibiotics.

Recently, the Word Health Organization has announced a global action plan on antimicrobial resistance and recommended avoiding the iatrogenic increase in drug-resistant bacteria.[27] Because many patients are being treated with eye drops for long periods, further surveys of bacterial resistance arising from these disinfectants in eye drops are required.

In conclusion, BAC-resistant *S. epidermidis* harboring the *qacC*/*smr* gene might be selected on the ocular surface exposed to eye drops containing BAC for more than 1 year.

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