Conjunctival Bacteria Flora of Glaucoma Patients During Long-Term Administration of Prostaglandin Analog Drops

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Purpose. To investigate the effects of the long-term use of prostaglandin analogs for glaucoma treatment on the indigenous flora of the conjunctiva.

Methods. Bacterial isolates were collected from the conjunctival sacs of 68 patients at Miyata Eye Hospital from February to September 2014, who had been receiving continuous monotherapy with prostaglandin analogs for glaucoma for at least 1 year. Minimum inhibitory concentrations of levofloxacin, gatifloxacin, moxifloxacin, cefmenoxime, tobramycin, chloramphenicol, and erythromycin against the isolates were measured to determine susceptibility.

Results. The positive culture rate in all cases was 90.5% (57/63 eyes), and a total of 79 bacterial strains were isolated. The isolated bacteria included aerobic gram-positive cocci (8% *Staphylococcus aureus* and 41% *Staphylococcus epidermidis*), coagulase-negative staphylococci (5%), *Streptococcus* spp. (1%), *Corynebacterium* spp. (4%), gram-negative bacteria (4%), and the facultative anaerobe *Propionibacterium acnes* (33%). The positive culture rates for patients using 0.005% latanoprost (Xa group) and 0.004% travoprost (Tz group) were 88.9% and 92.6%, respectively, with no statistically significant difference in the composition of isolated bacteria between groups. Methicillin-resistant *S. epidermidis* (MRSE) was significantly more frequently isolated in the Xa group. The antimicrobial susceptibility rates of *S. epidermidis* were significantly lower in the Xa group for levofloxacin, gatifloxacin, moxifloxacin, and tobramycin.

Conclusions. The indigenous flora may be affected by the long-term use of prostaglandin analogs. The higher incidence of MRSE in the Xa group should be considered during the long-term, continuous administration of eye drops, such as in glaucoma treatment.

Keywords: conjunctival bacteria flora, prostaglandin analogs, glaucoma medications

Currently, the only reliable, evidence-based method for treating glaucoma is decreasing the IOP.[1–3] Medical treatment with prostaglandin analogs or surgical procedures, such as trabeculectomy are most commonly used to decrease IOP.[4–7] In general, operative treatment is indicated for cases in which sufficient reduction of IOP cannot be achieved with medical treatment alone.[8] As the ultimate goal of glaucoma treatment is the maintenance of visual function for the rest of the patient's life, many patients undergo long-term administration of glaucoma eye drops.

Such repeated exposure of the ocular surface components such as the cornea and conjunctiva to the main agents and preservatives in eye drops may affect the corneal and conjunctival epithelia.[9,10] However, few studies have assessed the effects of these eye drops on the indigenous flora, which are considered important compositional elements of the conjunctiva. Thus, we investigated the effects of the long-term use of prostaglandin analogs for glaucoma treatment on the indigenous flora of the conjunctiva.

Methods

Subjects and Grouping

This prospective study was approved by the ethical review board of Miyata Eye Hospital and was conducted in accordance with the Declaration of Helsinki and ethical guidelines for clinical research. This study was registered in the UMIN Clinical Trials Registry (UMIN000019650). All subjects provided informed consent, and written permission was obtained.

Sixty-three glaucoma patients (63 eyes) and 44 healthy volunteers (44 eyes) were continuously enrolled in this study at Miyata Eye Hospital between February and September 2014. All patients had been receiving continuous monotherapy with prostaglandin analogs for glaucoma for at least 1 year. Exclusion criteria for the glaucoma patients were the use of eye drops other than prostaglandin analogs; history of glaucoma surgery; topical or systemic administration of antimicrobial agents in the past 2 weeks; history of severe side effects to fluoroquinolones; suspected bacterial, fungal, or viral infection; poorly controlled

ocular or systemic underlying disease; and presence of diseases. The inclusion criterion for healthy volunteers was no use of any eye drops or systemic antimicrobial agents within the last 3 months.

Patients had received one of the following medications: 0.005% latanoprost (Xalatan; Pfizer, Inc., New York, NY, USA) in 36 eyes (Xa group) or 0.004% travoprost (Travatan Z; Alcon Japan Co. Ltd., Tokyo, Japan) in 27 eyes (Tz group).

Specimen Collection

To collect specimens from the right eye, preservative-free oxybuprocaine hydrochloride (Minimus 0.4% eye drops; Senju Pharmaceutical Co. Ltd., Osaka, Japan) was used to anesthetize the convex surface of the lower conjunctival sac before scraping with a sterile cotton swab. The collected specimens were stored in a transport culture container (Anaport; The Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan) and transported to the Research Foundation for Microbial Diseases of Osaka University under refrigerated conditions (4°C–8°C).

Bacterial Culture and Isolation

For the culture, we used trypticase soy agar with 5% sheep blood, Columbia CNA agar with 5% sheep blood, MacConkey agar (Becton Dickinson Co., East Rutherford, NJ, USA), Columbia agar with 5% sheep blood (Becton Dickinson Co.), chocolate agar (Kyokutou Pharmaceutical Industrial Co. Ltd., Tokyo, Japan), and Sabouraud agar (EIKEN Co., Tokyo, Japan). Specimens were stored for 24 to 48 hours at 35°C (5% CO_2) in the trypticase soy agar with 5% sheep blood, Columbia CNA agar with 5% sheep blood, MacConkey agar, and chocolate agar. Further, specimens were stored at 37°C (80% N_2, 10% CO_2, and 10% H_2) for 1 to 5 days in the Columbia agar with 5% sheep blood, and at 30°C for 1 to 14 days in the Sabouraud agar. Thioglycolate medium (EIKEN Co.) was used as the enrichment culture, and specimens were stored at 35°C for 2 weeks. Bacteria identification was conducted using the API system (BioMerieux Industry, Tokyo, Japan).

Drug Susceptibility

For drug susceptibility testing, we measured the minimum inhibitory concentrations (MICs) of levofloxacin, gatifloxacin, moxifloxacin, cefmenoxime, ceftazidime, tobramycin, chloramphenicol, and erythromycin as target antimicrobial agents[11] against the obtained isolates. The antimicrobial susceptibility was determined based on Clinical and Laboratory Standards Institute standards at three levels: susceptible (S), intermediate (I), and resistant (R). The ratio of susceptible isolates to the overall susceptibility [S/(S + I + R)] was considered to represent the drug susceptibility. For *Staphylococcus epidermidis*, a MIC of oxacillin of less than or equal to 2 μg/mL was considered to indicate methicillin-susceptible *S. epidermidis* (MSSE), and a MIC of greater than or equal to 4 μg/mL was considered to indicate methicillin-resistant *S. epidermidis* (MRSE).

Statistical Analysis

For specimens obtained from each prostaglandin analog group and healthy controls, the bacterial positivity rate, distribution of bacterial isolates, MIC_{50}, MIC_{90}, and drug susceptibility rates were analyzed, which were compared among prostaglandin analogs and between the respective prostaglandin analogs and the control. The bacterial positive rate and drug susceptibility rates were analyzed using the Fisher exact test, and the logarithms of the MIC_{50} and MIC_{90} values were analyzed using Student's *t*-test. We further used logistic regression with drug duration administration as a covariate to consider the potential effects of different times of durations of the two types of drugs on the frequency of MRSE between the groups. The level of statistical significance was set at P less than 0.05.

Table 1. Patient Characteristics

	Xa Group, $n = 36$	Tz Group, $n = 27$	Ht Group, $n = 44$
Sex			
Male	20	11	11
Female	16	16	33
Age, y	68.4 ± 14.2	70.7 ± 12.7	47.9 ± 7.0
Administration period, mo (range)	82.9 ± 29.2* (12–251)	29.2 ± 15.9* (12–67)	–

* A statistically significant difference was noted in the administration periods between the Xa and Tz groups ($P < 0.01$) using the Student's *t*-test.

Results

Subject Characteristics

Table 1 shows the clinical and demographic characteristics of patients of each group. Of the 63 glaucoma patients (63 eyes) enrolled in this study, 34 were female, 20 were in the Xa group, and 11 were in the Tz group. The mean ages of patients in the Xa (68.4 ± 14.2 years) and Tz (70.7 ± 12.7 years) groups did not differ significantly; however, there was a statistically significant difference in the durations of administration between the two groups.

Of the 44 healthy volunteers (44 eyes), 33 were female. The mean age was 47.9 ± 7.0 years. There was a significant difference in the mean age between the healthy volunteers and glaucoma patients ($P < 0.05$).

Characterization of Bacterial Isolates

The positive culture rate from glaucoma patients was 90.5% (57/63 eyes). A total of 79 isolates from 57 eyes were identified. The majority of isolates consisted of aerobic gram-positive cocci, such as *S. aureus* (6 strains, 8%), *S. epidermidis* (32 strains, 41%), including MRSE (19 strains), other coagulase-negative staphylococci (CNS; 4 strains, 5%), and *Streptococcus* spp. (1 strain, 1%), as well as aerobic gram-positive bacilli, including *Corynebacterium* spp. (3 strains, 4%), gram-negative bacteria (3 strains, 4%), other gram-positive bacteria (4 strains, 5%), and facultative anaerobes, including *Propionibacterium acnes* (29 strains, 33%). Neither methicillin-resistant *S. aureus* nor fungi were detected.

The positive culture rate from the healthy control group was 84.1% (37/44 eyes). A total of 59 isolates from 37 eyes were identified, consisting of *S. epidermidis* (50.8%), including MRSE (10 strains), other CNS (6.8%), *Corynebacterium* spp. (5.1%), and *P. acnes* (35.6%); *S. aureus*, gram-negative bacteria, or fungi were detected.

Comparison of Bacterial Isolates Between the Xa and Tz Groups

The positive culture rates from the Xa and Tz groups were 88.9% (46 strains) and 92.6% (33 strains), respectively, with no

Table 2. Numbers of Isolated Strains

	Xa Group	Tz Group	Ht Group
No. of eyes	36	27	44
No. of positive culture eyes	32	25	37
S. aureus	4	2	0
MSSE	3	10	20
MRSE	16	3	10
S. caparae		1	
S. chromogenes	1		
S. hominis			1
S. lugdunennsis	1		3
S. warneri	1	1	4
S. oralis		1	
S. sp.			
Corynebacterium sp.	2	1	3
Propionibacterium acnes	15	11	21
Propionibacterium granulosum	1	2	
Propionibacterium sp.	1		
Klebsiella oxytoca		1	0
Morganella morganii		1	
Seratia sp.	1		
Total	46	33	59

significant difference in the distribution of bacterial isolates between groups (Table 2).

In the Xa and Tz groups, *S. epidermidis* was the most common isolate detected (40%), followed by *P. acnes*, *S. aureus*, and CNS in descending order (Table 2). We isolated four MSSE strains and 15 MRSE strains in the Xa group, and isolated 10 MSSE strains and three MRSE strains in the Tz group, indicating that MRSE was significantly more frequently isolated in the Xa group (Fig. 1). In addition, 20 MSSE strains and 10 MRSE strains were isolated in the healthy control group. Compared with the control group, the Xa group, but not the Tz group, had a significantly higher number of MRSE isolates.

We further evaluated the influence of the duration of use of Xa and Tz on the methicillin resistance rates of *S. epidermidis* using logistic regression with duration of use as a covariate. In this model, duration of use was not statistically significant (Fig. 2), and there was still a greater incidence of MRSE in the Xa group than in the Tz group (odds ratio = 11.66, 95% confidence interval = 1.79–76.08, $P = 0.0102$). This suggested that duration of use did not have a major influence on the methicillin resistant rate.

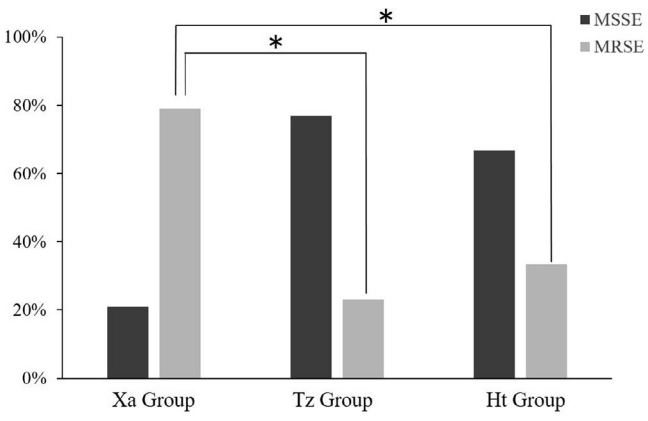

Figure 1. Comparison of the percentages of MSSE and MRSE strains detected in the Xa and Tz groups. *$P < 0.05$ (Fisher's exact test: Xa versus Tz, $P = 0.0033$; Xa versus Ht, $P = 0.0031$; Tz versus Ht, $P = 0.7203$); Ht, healthy control group.

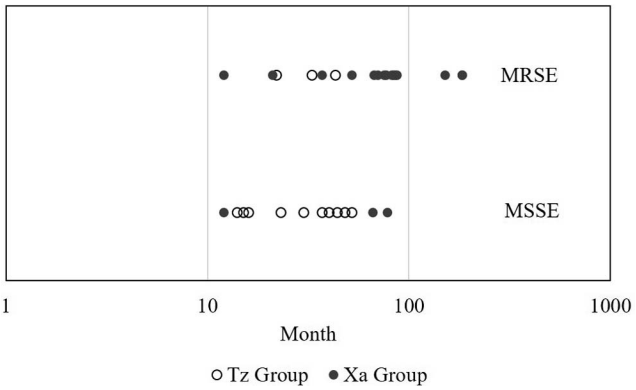

Figure 2. Distribution of the appearance of MSSE and MRSE detected in the Xa and Tz groups. Duration of use was added as a covariate to the logistic regression model, and did not have statistically significant influence ($P = 0.2202$).

The MIC_{50} and MIC_{90} values of levofloxacin, gatifloxacin, moxifloxacin, cefmenoxime, tobramycin, and erythromycin against the *S. epidermidis* isolates from the Xa group were significantly higher than those against isolates from the Tz group (Table 3). Accordingly, the *S. epidermis* susceptibility rates to levofloxacin, gatifloxacin, moxifloxacin, and tobramycin were significantly lower in isolates from the Xa group than in those of the Tz group (Table 4). There was no significant difference in the MICs against *S. epidermis* and drug susceptibility rates between the Tz group and healthy control group. The frequency of multiple-resistant *S. epidermidis*, showing resistance against more than three antibiotics among fluoroquinolones (one or more of levofloxacin, gatifloxacin, moxifloxacin), cephalosporins (one or more of ceftazidime and cefmenoxime), aminoglycosides (tobramycin), chloramphenicol, and macrolides (erythromycin) was 68% (13/19 strains) in the Xa group, 23% (3/13 strains) in the Tz group, and 27% (8/30 strains) in the healthy control group. Compared with the control group, the Xa group, but not the Tz group, had a significantly higher number of multiple-resistant *S. epidermidis* isolates ($P < 0.05$, Fisher exact test). We also compared the MIC of *S. epidermidis* in patients with respect to the duration of Xa by arbitrarily dividing these patients into two subgroups: Xa-1 and Xa-2, with less and more than a 67-month duration, respectively. There were no significant differences in the MICs to various antibiotics detected between the Xa-1 and Xa-2 groups (Table 5).

Discussion

The ultimate goal of glaucoma treatment is maintenance of visual function for the rest of the patient's life.[8] In many cases, glaucoma eye drops must be administered over a long period. Therefore, it is highly possible that minimal effects on the conjunctiva that would not be considered an issue with short-term administration could cause problems with long-term administration. To evaluate this possibility, we investigated the indigenous flora of the conjunctiva of patients that were prescribed prostaglandin analogs for glaucoma as monotherapy for at least 1 year, and found that the susceptibility of the indigenous flora to antimicrobial agents differed depending on the type of eye drops used. This suggests that the long-term use of glaucoma eye drops affects the indigenous flora of the conjunctiva.

Although the durations of use of Xa and Tz were both longer than 1 year with an average greater than 2 years, the duration of Xa use was significantly longer than that of Tz use.

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Table 3. Comparison of *S. epidermidis* MICs in the Xa and Tz Groups and Healthy Volunteer Groups

	Xa Group, $n = 19$		Tz Group, $n = 13$		*P* Value, (Xa vs. Tz)	Ht Group, $n = 30$		*P* Value, (Xa vs. Ht)
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}		MIC_{50}	MIC_{90}	
LVFX	4	128	≤0.25	4	0.0011	≤0.25	4	0.0022
(MIC range)	(≤0.25–128)		(≤0.25–4)			(≤0.25–16)		
GFLX	2	64	≤0.25	2	0.0011	≤0.25	2	0.0030
(MIC range)	(≤0.25–128)		(≤0.25–2)			(≤0.25–8)		
MFLX	1	32	≤0.25	1	0.0040	≤0.25	1	0.0120
(MIC range)	(≤0.25–64)		(≤0.25–1)			(≤0.25–4)		
CAZ	16	32	4	16	0.01013	4	16	0.0024
(MIC range)	(≤0.25–>32)		(≤0.25–32)			(≤0.25–16)		
CMX	8	16	0.5	4	0.0035	≤0.25	2	0.0001
(MIC range)	(≤0.25–32)		(≤0.25–4)			(≤0.25–8)		
TOB	8	128	≤1	64	0.0425	≤1	16	0.0016
(MIC range)	(≤1–128)		(≤1–128)			(≤1–64)		
CP	4	64	4	8	0.3512	4	8	0.2109
(MIC range)	(2–128)		(2–64)			(4–8)		
EM	32	>64	≤0.25	32	0.0325	0.5	>64	0.0325
(MIC range)	(≤0.25–>64)		(≤0.25–>64)			(≤0.25–>64)		

MIC, minimal inhibitory concentration; LVFX, levofloxacin; GFLX, gatifloxacin; MFLX, moxifloxacin; CAZ, ceftazidime; CMX, cefmenoxime; TOB, tobramycin; CP, chloramphenicol; EM, erythromycin.

Therefore, this difference raised the possibility that the longer administration of eye drops with more visits to eye clinics could influence the incidence of MRSE. However, the results of logistic regression correcting for the duration of drug administration suggested that this difference between groups did not have a substantial influence on the overall findings, and there was no difference in MICs to various antibiotics between patients taking Xa for less than or more than 67 months. Therefore, it appears that the use of Xa for longer than 1 year might be sufficient to cause changes in the antibiotic susceptibility of *S. epidermidis*. Together, these results suggest that the ocular surface flora can be influenced and changed relatively early after the administration of glaucoma eye drops. To further validate this hypothesis, it will be necessary to conduct a prospective clinical study to directly evaluate the influence of the duration use of glaucoma eye drops on the ocular surface flora.

Specifically, the antimicrobial susceptibility of isolates from the conjunctival sac was lower in eyes administered Xa than in eyes administered Tz and those of healthy volunteers, suggesting that Xa induces the antimicrobial resistance of micro-organisms. One of the key differences between these eye drops is the presence or absence of the additive benzalkonium chloride (BAC). In Xa, 0.02% BAC is included as a preservative,[12] whereas a zinc-based ion buffer system is included in Tz instead of BAC.[13] We believe that this one difference in preservative choice might have influenced our results. However, many ingredients other than preservatives are added to the eye drop products. Therefore, further studies using the drug itself or the basic solutions comprising each product are needed to clarify their effects on conjunctival microflora.

Table 4. Comparison of *S. epidermidis* Susceptibility Rates Between the Xa and Tz Groups

	Xa Group	Tz Group	*P* Value (Xa vs. Tz)	Ht Group	*P* Value (Xa vs. Ht)
LVFX	21.1	76.9	0.0033	70.0	0.0012
GFLX	21.1	76.9	0.0033	70.0	0.0012
MFLX	26.3	76.9	0.0105	70.0	0.0038
CAZ	26.3	69.2	0.0293	86.7	0.0002
CMX	84.2	100.0	0.2523	100.0	0.0526
TOB	36.8	76.9	0.0359	83.3	0.0017
CP	78.9	92.3	0.6247	100.0	0.0185
EM	31.6	69.2	0.0702	50.0	0.2467

The main aim of adding preservatives to eye drops is to prevent potential contamination within the eye drop container. As BAC has excellent antibacterial properties and long-term stability, it is widely used as a preservative.[14–16] It was originally thought that the use of antiseptics such as BAC would not lead to drug resistance if used at realistic concentrations, unlike antimicrobial agents, in which inappropriate drug selection or concentration may destroy sensitive bacteria only, leading to the selection of drug-resistant bacteria. Moreover, as antiseptics kill microbes through a combination of mechanisms, such as albuminous degeneration of the cell membrane, enzyme inhibition, and increased membrane permeability, the likelihood of their selecting for bacterial resistance is low. Nevertheless, it has been reported that when antiseptics are used at much lower than realistic concentrations, bacterial resistance to the antiseptic may develop.[17]

The concentration of BAC in 0.005% latanoprost is 0.02%, which is higher than the bacterial MIC, and has been demonstrated to effectively prevent contamination within the container.[18] Thus, it appears that the appropriate concentration has been achieved within this formulation. However, Nakamura et al.[19] reported that after the administration of eye drops containing BAC, the BAC was quickly diluted by the lacrimal fluid; therefore, its concentration decreased over time. Specifically, 5 minutes after the eye drops were administered, BAC decreased to 1/10 or lower of its original concentration, and continued to slowly decrease thereafter. Thus, it is likely that indigenous flora on the conjunctiva may be exposed to lower than appropriate concentrations of this antiseptic.

In recent years, it has been suggested that resistant genes may be acquired or may mutate in bacteria that exhibit strong resistance to antiseptics. In such cases, the mechanism for resistance is known to be the promotion of discharge of the antiseptic outside the bacterial cells.[20] Because there is no drug specificity associated with this discharge mechanism, bacteria resistant to antiseptics can also readily acquire cross-resistance to antimicrobial agents.[21,22] Accordingly, a possible explanation for the observed lower susceptibility to antimicrobial

Table 5. Comparison of the MICs of *S. epidermidis* Between the Shorter-Term and Longer-Term Xa Groups With Those of the Tz Group

	Xa-1, $n = 7$	Xa-2, $n = 12$	*P* Value, (Xa-1 vs. Xa 2)	Tz, $n = 13$	*P* Value, (Xa-1 vs. Tz)	*P* Value, (Xa-2 vs. Tz)
Administration period, mo	12–67	70–251		12–67		
LVFX	4.88	2.83	0.5657	0.50	0.0252	0.0101
GFLX	2.44	1.59	0.6199	0.38	0.0432	0.0096
MFLX	1.35	1.00	0.7079	0.34	0.0920	0.0193
CAZ	10.77	13.45	0.7676	5.51	0.3406	0.1259
CMX	2.44	4.00	0.5275	0.69	0.1053	0.0051
TOB	19.50	5.66	0.1541	2.35	0.0222	0.2101
CP	9.75	6.35	0.5435	5.22	0.3598	0.6270
EM	11.89	8.00	0.7654	1.17	0.0855	0.0812

P values were calculated using Fisher's exact test and Student's *t*-test.

agents in the eyes of patients using Xa than in the eyes of patients using Tz could be the development of cross-resistance due to BAC resistance. However, further research is required to confirm the actual cause of this difference, because the influence of the base components or main agents other than BAC cannot be ruled out.

One of the limitations of this study was that we were not able to recruit healthy volunteers of the same age group as the glaucoma patients for this study because it was difficult to find subjects with healthy eyes that had not used any eye drops such as dry eye therapeutic eye drops and those for cataract prevention who were in the same age group as the glaucoma patients. However, we previously reported that 48.3% (3084/6391) of *S. epidermis* isolates from the conjunctival sac of patients (more than 60-years old: 87.9%) before undergoing ophthalmic surgery at Miyata Eye Hospital from 2008 to 2015 were methicillin-resistant.[23] Hori et al.[24,25] reported that methicillin-resistant CNS (MRCNS), including *S. epidermidis*, accounted for 53.8% and 37.8% of the total CNS isolates in dry eye patients (mean age 60.3 years) and patients undergoing cataract surgery (mean age 66.3 years), respectively. In addition, Hsu et al.[26] reported that MRCNS, including *S. epidermidis*, accounted for 45.2% of the isolates obtained from patients undergoing cataract surgery. It is generally considered that the rate of MRSE in *S. epidermidis* isolates increases with advanced age. In this study, MRCNS, including *S. epidermidis*, of the Xa group accounted for 78.9% of the isolates, which is a higher rate than that observed in previous studies in the context of other diseases, such as before cataract surgery and dry eye. This result suggests that specimen culture and antimicrobial susceptibility tests may need to be performed before conducting eye surgery, especially glaucoma surgery, to determine the most appropriate treatment.

Overall, the results of this study suggest that the long-term use of prostaglandin analogs for glaucoma may affect the resistance of indigenous flora on the conjunctiva. As there are no reports on clinical problems associated with such resistance to date, we do not believe that these findings are of great significance when determining treatment strategies. Nevertheless, our results raise concern for potentially selecting for causative microbes of bleb-related infection after trabeculectomy. In our study, we did not exclude eyes with filtration blebs. In some cases, even after filtration surgeries, glaucoma eye drops are added to further lower the IOP in eyes with blebs. This additional treatment may change the indigenous flora of such eyes, and resistant microbes against the standard antibiotic agents may reside on the ocular surface, which may induce bleb-related infection. Thus, it may be important to periodically monitor the indigenous flora in postoperative glaucoma eyes treated with eye drops, not only for IOP reduction but also for the treatment of other ocular diseases.

In conclusion, we found that the indigenous flora may be affected by the long-term use of prostaglandin analog products. In particular, the frequency of MRSE isolates increased more in the Xa group than in the Tz group. This tendency should be considered during the long-term, continuous administration of eye drops, such as for the treatment of glaucoma, to prevent selecting for resistant strains that could lead to uncontrollable infection.

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