• Clinical Research •

Shifting hierarchy of the conjunctival florae in the patients employed a long-time topical fluoroquinolone

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Abstract

● **AIM:** To observe the shifting hierarchy of the conjunctival florae in the patients who employed a long-time topical fluoroquinolone and characterize the consequent variations of their antibiotic sensitivity and virulence.

● **METHODS:** A total of 143 eyes (143 patients) who suffered from the non-infectious corneal ulcer and topically used fluoroquinolone more than 2wk were enrolled as the fluoroquinolone eye. The untreated fellow eye was considered as the contralateral eye. Seventy-five healthy subjects were selected as the control. The culture positivity and strains of the isolated conjunctival florae were observed. Their antibiotic susceptibility and expression of the virulence-related genes were detected.

● **RESULTS:** Florae were recovered from 84.0%, 37.1%, and 57.3% of the conjunctival swabs in the control, fluoroquinolone eye, and contralateral eye, respectively. The most frequently isolated microorganisms were *Staphylococcus epidermidis* (34.9%) in the control, followed by *Staphylococcus aureus* (17.5%), *Staphylococcus saprophyticus* (14.3%), *Micrococcus* (9.5%), *Propionibacterium acnes* (7.9%). However, those orderly ranks shifted to *Staphylococcus aureus* (34.0%), *Propionibacterium acnes* (20.8%), *Candida albicans* (17.0%), *Pseudomonas aeruginosa* (9.4%) in the fluoroquinolone eye. A growing number of the fluoroquinolone-resistant florae survived in the fluoroquinolone eye, accompanied by an increased expression of the virulence-related genes.

● **CONCLUSION:** A long-time topical fluoroquinolone leads to a shifting hierarchy of the conjunctival florae, accompanied by the consequent variations of the antibiotic sensitivity and virulence.

● **KEYWORDS:** fluoroquinolone; conjunctival flora; antibiotic sensitivity; virulence; corneal ulcer
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INTRODUCTION

The term for "normal microbial flora" refers to a population of microorganisms which dwell within the intestinal tract, oral cavity, throat duct and conjunctival sac of the healthy individuals[1]. Those microorganisms interact with the others and host to drive coevolution, inhibit pathogen overgrowth, and shape a specific hierarchy of the local microbial flora[2]. The accumulating clinical shreds of evidence confirmed that the disbalance of normal microbial flora after using of wide-spectrum antibiotics irritates their pathogenicity and resulted in dysbacteriosis, suggesting that the community context in which those microorganisms lived was an important factor affected the variable virulence[3].

The conjunctival sac is rich in nutrients and, consequently, inherently supports a diverse range of microorganisms which constitute the normal conjunctival florae[4]. The conjunctival florae are acquired at birth and remain a specific hierarchy throughout the whole life, which may show a slight variation depending on the environmental factors, age, season, immunity, and general hygienic conditions[5]. Under the normal circumstances, the conjunctival florae maintain the balance or homeostasis between themselves and inhibit the survival of more pathogenic species[6]. Fluoroquinolone is widely used for the prophylaxis of the conjunctival flora-associated infection, which shows the bactericidal activity against both Gram-negative and -positive bacteria. However, some questions come to the clinician. Did the community of the conjunctival florae change after a long-time topical fluoroquinolone and, if yes, what change would happen? What was the consequent effect of the changing florae on their antibiotic sensitivity and virulence? In this study, we characterized the shifting hierarchy of the conjunctival florae in the patients who employed a long-time topical fluoroquinolone and observed the variable

antibiotic sensitivity and virulence-related gene expression of those florae.

SUBJECTS AND METHODS

Ethical Approval The study was followed the tenets of the Declaration of Helsinki and approved by the Institutional Ethics Board of the Third Affiliated Hospital, Guangzhou Medical University. Written informed consents were obtained from all the subjects.

Study Subjects This study enrolled a total of 143 eyes (143 patients) during the period from January 2013 to December 2017, who suffered from a non-infectious corneal ulcer caused by acid/base chemical injury, neurotrophic keratopathy, or exposing, and topically used fluoroquinolone more than 2wk for the prophylaxis of the conjunctival flora-associated infection. The fluoroquinolone-treated eyes were named as the fluoroquinolone eye but the untreated contralateral eyes as the contralateral eye. They consisted of 109 males and 34 females, mean age 47.3±17.7y (range from 19 to 68y). Exclusion criteria included: 1) primary bacterial or fungal ocular infection; 2) history of ocular surgery within 3mo; 3) suffered from diabetes, local or systemic immunological diseases; 4) use of other topical or systemic antibiotics within the last 3mo. Seventy-five healthy peoples, matching age and gender, were selected as the control. None of them topically or systemically used medications.

Sample Collection Ocular specimen for bacterial analysis was collected from the upper, lower, and fornix bulbar conjunctiva using a disposable sterile dry absorbent cotton swab without anesthesia. It cannot be too careful to avoid contacting with eyelids and eyelashes lest the sample was contaminated. A clean cotton swab was considered as blank control.

Bacterial Culture The collected samples were plated onto blood agar and MacConkey agar culture media, incubated with 5% CO_2 at 37℃ and observed at 24, 48 and 72h, which was absent of bacterial growth after 72h was considered a negative result. For the anaerobic culture, the swab was rolled onto an anaerobic blood agar containing nutrient broth. The plate was immediately sealed in a sterile air-locked plastic bag and incubated at 37℃ for at least 7d. Subsequently, the morphological assessment, Gram-staining, and quantitative real-time reverse transcription-polymerase chain reaction PCR (qRT-PCR) were employed to identify the bacterial strain.

Fungal Culture The swab was plated on Sabouraud dextrose agar supplemented with chloramphenicol (0.05%), incubated with 5% CO_2 at 27℃ for 14d, and observed weekly for fungal growth. Fungi were identified to the genus level by microscopic examination of a wet mount of fungal colonies using lactophenol cotton blue stain and qRT-PCR. Fungi that could not be initially identified because they lacked typical characteristics were subcultured on Sabouraud dextrose agar and observed for an additional 21d.

Antibiotic Susceptibility Assay To determine antibiotic susceptibility of the isolated strains, the minimum inhibitory concentration (MIC) susceptibility of them to levofloxacin, penicillin, tobramycin, tetracycline, rifampicin, and vancomycin was observed using the routine disc diffusion and/or microdilution method according to the National Committee for Laboratory Standards guidelines.

Detection of the Virulence-Related Genes In order to observe the variable virulence of the conjunctival florae isolated from the control, contralateral eye, and fluoroquinolone eye, the genes encoding the virulence-related factors were detected by qRT-PCR, including mecA, toxic shock syndrome toxin-1 (TSST-1), Panton-Valentine leukocidin (PVL), and phenol-soluble modulins-alpha (PSM-α) for *Staphylococcus*, exoU, exoS, exoY, and exoT for *Pseudomonas aeruginosa*, Christie-Atkins-Munch-Peterson (CAMP) for *Propionibacterium acnes*, and ALS2, ALS5, SAP1, SAP2, and SAP3 for *Candida Albicans*. The primers used in this study were listed in Table 1. The amplification condition was as follows: 5min at 94℃, then 35 cycles of 30s at 94℃, 30s at 55℃, and 30s at 72℃; followed by a final elongation step of 5min at 72℃ (Table 1)[7-10].

Statistical Analysis Quantitative data were expressed as mean±SD. Descriptive values of data were computed as percent frequencies. Chi-square test or ANOVA was applied to compare the study parameters between the control, fluoroquinolone eye, and contralateral eye. $P<0.05$ was considered a statistical difference.

RESULTS

Demographic Characteristics of the Enrolled Patients The demographic characteristics of the studied subjects were presented in Table 2. There was no significant difference in age and gender between the control, fluoroquinolone eye, and contralateral eye ($P>0.05$).

Shifting Hierarchy of the Isolated Conjunctival Florae The microorganism profiles isolated from the control, fluoroquinolone eye, and contralateral eye were shown in Table 3. Microflorae were recovered from 84.0%, 37.1%, and 57.3% of the conjunctival swabs in the control, fluoroquinolone eye, and contralateral eye, respectively, which did show a statistical difference between the three groups ($P=0.041$). The most frequently isolated microflora in the control was *Staphylococcus epidermidis* (34.9%), followed by *Staphylococcus aureus* (17.5%), *Staphylococcus saprophyticus* (14.3%), *Micrococcus* (9.5%), *Propionibacterium acnes* (7.9%). However, those orderly ranks shifted to *Staphylococcus aureus* (34.0%), *Propionibacterium acnes* (20.8%), *Candida albicans* (17.0%), *Pseudomonas aeruginosa* (9.4%) in the fluoroquinolone eye. The culture positivity of *Propionibacterium acnes* (10.8%), *Candida albicans* (7.5%),

Table 1 Primers used for amplification of the virulence-related genes

Primers	Oligonucleotide sequence (5'-3')	Sizes (bp)	Specificity	Reference
mecA-F	ACTGCTATCCACCCTCAAAC	147	mecA	[7]
mecA-R	CTGGTGAAGTTGTAATCTGG			
PVL-F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	PVL	[8]
PVL-R	GCATCAASTGTATTGGATAGCAAAAGC			
TSST-1-F	ACCCCTGTTCCCTTATCATC	326	TSST-1	[8]
TSST-1-R	TTTTCAGTATTTGTAACGCC			
PSM-α-F	CGATGTGGTCAGTTTGATCGG	377	PSM-α	Designed
PSM-α-R	GCCTAGCCCAGCCAGTTAAG			
exoU-F	CCA TCGTTGGGGGCTACTGCCTCCT	830	exoU	[9]
exoU-R	TGGGGAATGTAAGCACTCAACCGAT			
exoS-F	TCAGGTACCCGGCATTCACTACGCGG	550	exoS	[9]
exoS-R	TCACTGCAGGTTCGTGACGTCTTTCTTTTA			
exoT-F	TCAGCAGAACCCGTCTTTCGT	407	exoT	[9]
exoT-R	GCCAGGCGCGTGTGATCCTTC			
exoY-F	ACCATGCGTATCGACGGTCATC	323	exoY	[9]
exoY-R	TTGCTGAGATGCTGGTCGACAC			
CAMP-F	TCTTCCCGCACTGTGTCTTC	198	CAMP	[10]
CAMP-R	TCTCAAACCAGGCTCAACCC			
ALS2-F	CAGAGCGACGGGAAGAGTTT	415	ALS2	Designed
ALS2-R	CCGGGTCATCAAGGACGTAA			
ALS5-F	CCATCACCAACGGTCCAGAA	833	ALS5	Designed
ALS5-R	TGGCTCCCCTGCTATAGTGT			
SAP1-F	TGCTGCCACTGGACAAATCA	379	SAP1	Designed
SAP1-R	AGGTTGACCGTTAGCGTAGC			
SAP2-F	ATGCTGCCACGGGACAAATA	347	SAP2	Designed
SAP2-R	TTCGGAAGCTGGAACGGAAA			
SAP3-F	ACCAACGTCAACGTCAAGAGA	356	SAP3	Designed
SAP3-R	TCGGCAAATTGTTGCTTTGTG			

Table 2 Demographic and clinical information of the enrolled subjects

Parameters	*n*	Age (mean±SD)	M/F	Diagnosis				
				HE	AC	BA	NK	EX
Fluoroquinolone eye	143	47.3±17.7	109/34	0	39	69	23	12
Contralateral eye	143	47.3±17.7	109/34	143	0	0	0	0
Control	75	43.5±14.3	55/20	75	0	0	0	0

M: Male; F: Female; HE: Healthy; AC: Acid chemical injury; BA: Basic chemical injury; NK: Neurotrophic keratopathy; EX: Exposing.

Pseudomonas aeruginosa (3.2%) slightly increased in the contralateral eye, compared with those in the control.

Variable Antibiotic Susceptibility of the Isolated Conjunctival Florae The tested antibiotics sensitivity of the isolated conjunctival florae was shown in Figure 1. With regard to Gram-positive *Staphylococcus* strain, the results of sensitivity assay to levofloxacin, penicillin decreased to 33.3% and 4.6% in the fluoroquinolone eye and to 46.7% and 66.7% in the contralateral eye, which was lower than 85.0% and 93.3% in the control, respectively (P<0.0001). However, the sensitivity to rifampicin was 26.7% in the fluoroquinolone eye, which was higher than 13.3% in the control (P=0.0203).

To *Pseudomonas aeruginosa*, their sensitivity to levofloxacin, tobramycin, and tetracycline decreased to 46.7%, 20.0%, and 6.7% in the fluoroquinolone eye and 61.9%, 47.5%, and 13.3% in the contralateral eye, respectively, which was lower than 86.7%, 80.0%, 40.0% in the control (P<0.0001). The sensitivity to rifampicin increased to 86.7% in the fluoroquinolone eye, compared with 66.7% in the control (P=0.0091).

The sensitivity of *Propionibacterium acnes* to levofloxacin, tobramycin, and vancomycin decreased to 40.0%, 53.3%, 46.7% in the fluoroquinolone eye and to 78.5%, 78.5%, and 71.4% in the contralateral eye, respectively, which was lower than 93.3%, 93.3%, and 80.0% in the control (P<0.0001).

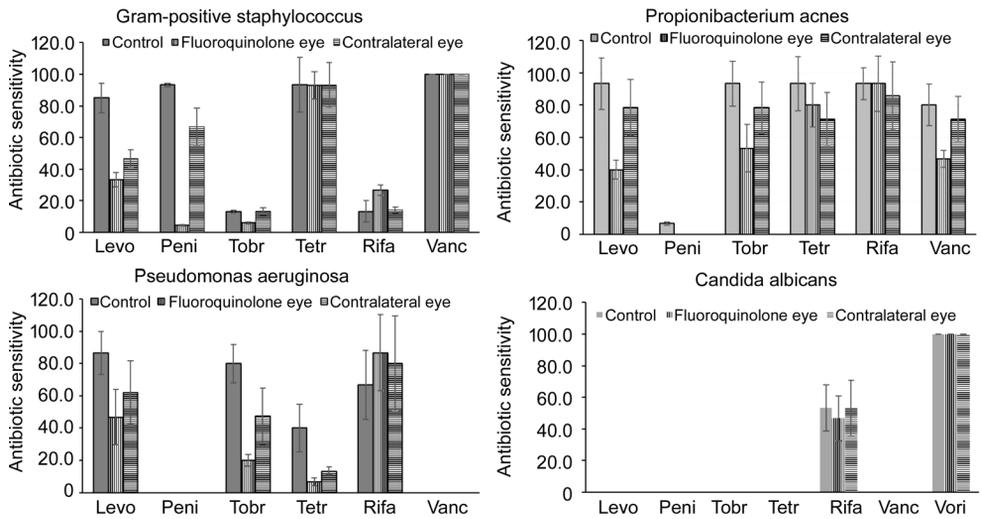

Figure 1 The antibiotic sensitivity of the isolated conjunctival florae Levo: Levolevofloxacin; Peni: Penicillin; Tobr: Tobramycin; Tetr: Tetracycline; Rifa: Rifampicin; Vanc: Vancomycin; Vori: Voriconazole.

Table 3 Microflora profiles of the isolated conjunctival florae *n* (%)

Parameters	Control eye	Fluoroquinolone eye	Contralateral eye	*P*
Staphylococcus epidermidis	22 (34.9)	4 (7.5)	25 (26.9)	0.0022[a]
				0.0004[b]
Staphylococcus aureus	11 (17.5)	18 (34.0)	22 (23.7)	0.1165[a]
				0.0409[b]
Staphylococcus saprophyticus	9 (14.3)	3 (5.7)	8 (8.6)	0.2650[a]
				0.1286[b]
Micrococcus	6 (9.5)	2 (3.8)	5 (5.4)	0.3993[a]
				0.2234[b]
Propionibacterium acnes	5 (7.9)	11 (20.8)	10 (10.8)	0.0916[a]
				0.0461[b]
Streptococcus viridans	4 (6.3)	0	4 (4.3)	0.1965[a]
				0.0619[b]
Diplococcus pneumoniae	2 (3.2)	0	4 (4.3)	0.3215[a]
				0.1907[b]
Pseudomonas aeruginosa	1 (1.6)	5 (9.4)	3 (3.2)	0.0918[a]
				0.0537[b]
Pseudomonas fluorescens	1 (1.6)	1 (1.9)	2 (2.2)	0.9686[a]
				0.9018[b]
Candida albicans	1 (1.6)	9 (17.0)	7 (7.5)	0.0100[a]
				0.0033[b]
Unidentified	1 (1.6)	0	3 (3.2)	
Total	63	53	93	

[a]A statistical analysis among control, fluoroquinolone eye, and contralateral eye; [b]A statistical analysis between control and fluoroquinolone eye.

Candida albicans showed 100% sensitivity to voriconazole in the control, fluoroquinolone eye, and contralateral eye. However, it was almost resistant to all the antibiotics except rifampicin. The results of the sensitivity assay were 53.3%, 46.7%, and 53.3% to rifampicin in the control, fluoroquinolone eye, and contralateral eye, respectively (P=0.52).

Variable Expression of the Virulence-Related Genes of the Isolated Conjunctival Florae The variable expression of the virulence-related genes was presented in Table 4 and Figure 2. To the detected expression of mecA, TSST-1, PVL, and PSM-α among the isolated *Staphylococcus*, the total positivity was 7.7%, 31.5%, 14.5% in the control, fluoroquinolone eye,

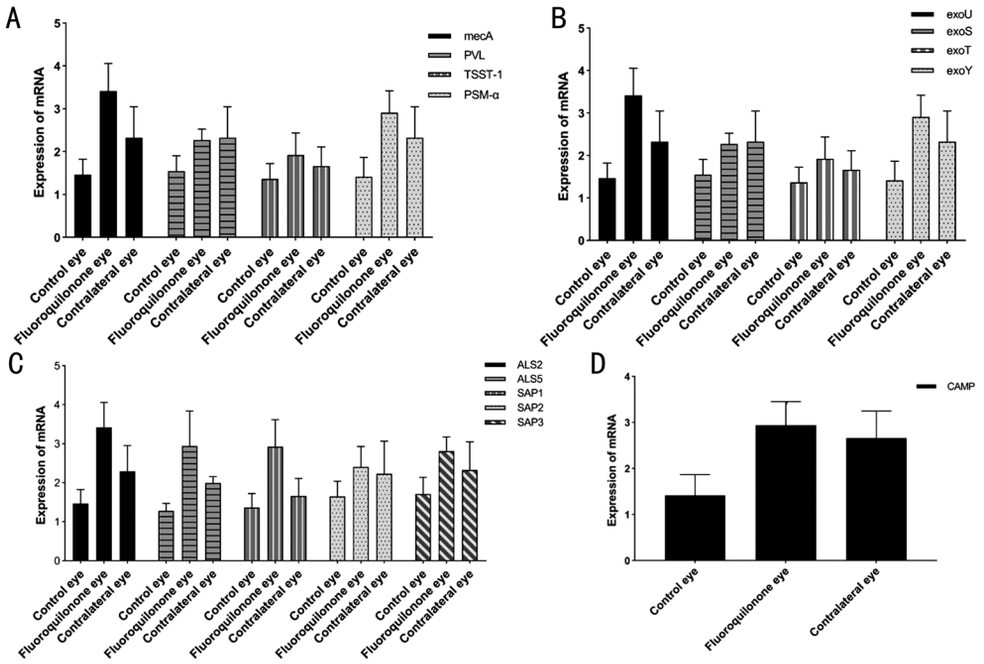

Figure 2 The expression level of the virulence-related genes A: The expression of mecA, PVL, TSST-1, and PSM-α in *Staphylococcus*; B: The expression of exoU exoS, exoT, and exoY in *Pseudomonas aeruginosa*; C: The expression of ALS2, ALS5, SAP1, SAP2, and SAP3 in *Cadida albicans*; D: The expression of CAMP in *Propionibacterium acnes.*

Table 4 The positive expression of the virulence-related genes in the isolated conjunctival florae

Parameters	Control	Fluoroquinolone eye	Contralateral eye	*P*
Staphylococcus				
mecA	4	8	13	
PVL	1	3	3	
TSST-1	2	3	5	
PSM-α	6	15	11	
Total	13/168 (7.7%)	23/92 (31.5%)	32/220 (14.5%)	0.0007
Pseudomonas aeruginosa				
exoU	0	3	2	
exoS	1	4	2	
exoT	0	1	0	
exoY	1	4	3	
Total	2/4 (50.0%)	12/16 (75.0%)	7/12 (58.3%)	0.0129
Cadida albicans				
ALS2	1	10	6	
ALS5	1	10	5	
SAP1	1	10	7	
SAP2	0	9	7	
SAP3	0	9	6	
Total	3/5 (60.0%)	48/50 (96.0%)	31/35 (88.6%)	0.0210
Propionibacterium acnes				
CAMP	2	8	10	
Total	2/5 (40.0%)	8/8 (100.0%)	7/10 (70.0%)	0.0027

and contralateral eye, respectively, which showed a significant difference between the three groups. Correspondingly, the increased expression of mecA and PSM-α was also seen in the fluoroquinolone eye and contralateral eye, compared with that in the control (P=0.0161 and 0.0487, respectively).

For the isolated *Pseudomonas aeruginosa,* there was a significant difference in the total positivity of the detected gene expression of exoU, exoS, exoY, and exoT between the control, fluoroquinolone eye, and contralateral eye, presented as 50.0%, 75.0%, and 58.3%, respectively. However, exoU and exoY showed the increased expression in the fluoroquinolone eye and contralateral eye, compared with those in control (P=0.0196 and 0.0311, respectively).

The detected expression of genes encoding CAMP in *Propionibacterium acnes* was positive for 100% of the fluoroquinolone eye, 70% of the contralateral eye, and 40% of the control. The expression of CAMP was increased in the fluoroquinolone eye and contralateral eye, compared with that in the control (P=0.0255).

For the isolated *Candida albicans*, the results obtained for ALS2, ALS5, SAP1, SAP2, and SAP3 positive expression were 94.0% and 88.4% in the fluoroquinolone eye and contralateral eye, respectively, and higher than 60.0% in the control. The increased expression of ALS2, ALS5, and SAP1 appeared in the fluoroquinolone eye and contralateral eye, compared with that in the control (P=0.0284, 0.0209, and 0.0320, respectively).

DISCUSSION

The conjunctival florae develop at birth and vary throughout the whole life[11]. Due to the discrepant sensitivity of the conjunctival florae, topical fluoroquinolone may inhibit the growth of some antibiotic-sensitive florae but allow the survival and overgrowth of other antibiotic-resistant florae, which resulted in the imbalanced ocular surface florae[12-13].

A better understanding of the change of the conjunctival florae after a long-time topical fluoroquinolone is helpful in the prophylaxis, diagnosis, and treatment of the opportunistic infection and dysbacteriosis associated with the conjunctival florae.

Shifting Hierarchy of the Isolated Conjunctival Florae In this study, *Staphylococcus epidermidis* and *Staphylococcus aureus* are the most common florae dwelling in the conjunctival sac in the healthy control, accompanied by a small number of anaerobes and fungi, which was consistent with the previous reports[14-16]. Although the total culture positivity of the isolated conjunctival florae decreased after a long-time topical fluoroquinolone, it resulted in the especial increase of the single culture positivity, including *Staphylococcus aureus, Propionibacterium acnes, Pseudomonas aeruginosa*, and *Candida albicans.* The absolute, not percentage, increase of those florae was in large part due to the disruption of the counterbalance in the conjunctival microbial community. It suggested that the long-time topical fluoroquinolone resulted in a shifting hierarchy of conjunctival florae, characterized by inhibition of the fluoroquinolone-sensitive microflorae but a concomitant overgrowth of the fluoroquinolone-resistant ones.

Variable Antibiotic Susceptibility of the Isolated Conjunctival Florae Corresponding to the shifting hierarchy of the conjunctival florae, the concomitant variation of the antibiotic susceptibility had been seen in the survival microorganisms.

The assay of antibiotic susceptibility showed a growing number of fluoroquinolone resistance in the florae isolated from the fluoroquinolone eye, including *Staphylococcus aureus, Propionibacterium acnes, Pseudomonas aeruginosa*, although most of which were sensitive to fluoroquinolone under the normal condition. It has been widely accepted that there is the positive relationship between increasing fluoroquinolone use and fluoroquinolone-resistance[17]. Furthermore, it was interesting to note that the conjunctival florae isolated from the fluoroquinolone eye also showed a decreased sensitivity to other types of antibiotics, such as penicillin, tobramycin, tetracycline. It suggested that the shifting hierarchy of conjunctival florae or, in other words, the changing microbial community contributed to the development of the antibiotic resistance of the florae. However, in this study, most conjunctival florae isolated from fluoroquinolone eye showed good sensitivity to rifampicin, which corresponded to the treating responses of rifampicin in some clinical cases (not shown). It gave us a reminder that the shifting conjunctival florae resulted in the variable antibiotic sensitivity and, consequently, required a corresponding modification of clinical therapeutic schedule in those patients who employed a long-time topical fluoroquinolone.

Virulence-Related Gene Expression of the Isolated Conjunctival Flora The growing shreds of evidence implied that the fluoroquinolone disproportionately increased the risk of infection with antibiotic-resistant bacteria[18]. Whether was the increased infectious risk associated with the virulence-related genes of the antibiotic-resistant bacteria? mecA, TSST-1, PVL, and PSM-α have been proposed as virulence factors for staphylococcus, which were closely associated with its antibiotic resistance and clinical scenarios[19-20]. *Pseudomonas aeruginosa* secretes four known effectors: exoS, exoT, exoU, and exoY and shows their cytotoxicity both *in vitro* and *in vivo* assays[21-22]. The CAMP is a virulence factor for *Propionibacterium acnes* and its neutralized antibody is chosen as a therapeutic target for *Propionibacterium acnes* related infection[10,23]. The major virulence factors that mediate the pathogenesis of *Candida albicans* include ALS2, ALS5, SAP1, SAP2, and SAP3[24-25]. In this study, the expression of most if not all virulence-related genes increased in the conjunctival florae isolated from the fluoroquinolone eye, which suggested that the shifting hierarchy of the conjunctival florae could contribute to the increased virulence and opportunistic pathogenicity of *Staphylococcus, Pseudomonas aeruginosa, Propionibacterium acnes,* and *Candida albicans.* It is a real fact that fungal spores and gram-negative bacillus were found in the primary non-infectious corneal ulcer in patients who employed a long-time topical fluoroquinolone (not shown).

In addition, the conjunctival florae isolated from the untreated contralateral eye showed a similar and non-significant changing trend in the culture positivity, antibiotic sensitivity, and expression of the virulence-related genes. The real mechanism for it remains unclear. Whether there is a neuroendocrine pathway to mediate the crosstalk of the microflorae between the fluoroquinolone eye and the contralateral eye needs to be further clarified. However, it is a clue that the effect of the shifting hierarchy of the conjunctival florae might radiate to the adjacent area but not limited to local.

In conclusion, the long-time topical fluoroquinolone might lead to a shifting hierarchy of the conjunctival florae, characterized by the overgrowth of the fluoroquinolone-resistant microflora with increased antibiotic resistance and expression of virulence-related genes. The molecular mechanism behind them needs to be further explored. However, it is worth to note that the shifting conjunctival flora provides a chance for opportunistic pathogens and subsequent opportunistic infection. More works need to do to find the best strategy for the use of antibiotics in the clinical prophylaxis of the conjunctival flora-associated infection.

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