• Clinical Research •

Conjunctival microbiota variations in a subset of middle-aged and elderly individuals from Beijing, China

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Abstract

• **AIM:** To isolate and identify the conjunctival microbiota of cataract patients and analyze the associated influencing factors.

• **METHODS:** This study recruited 216 participants (216 eyes) from April 2022 to July 2022. Under the condition of no antibiotic use prior to cataract surgery, sterile swabs were used to collect samples from the lower conjunctival sac. Bacterial cultures were then conducted, followed by species identification through 16S rDNA gene sequencing. Clinical factors associated with positive or negative bacterial isolation rates were analyzed, including age, gender, meibomian gland dysfunction (MGD), history of hypertension, history of diabetes, history of cancer, history of infectious diseases and the habit of wearing masks.

• **RESULTS:** Among the 216 eyes, 78 eyes yielded isolates, with an isolation rate of 36.11%, detecting a total of 122 strains. Gram-positive rods accounted for 49.18% (60 strains), gram-positive cocci accounted for 45.08% (55 strains), gram-negative bacteria accounted for 4.92% (6 strains), and fungi accounted for 0.82% (1 strain). This study found that the most abundant genera in the conjunctival sac were *Corynebacterium* (42.62%), *Staphylococcus* (31.15%), *Micrococcus* (9.84%), *Acinetobacter* (4.10%), and *Bacillus* (3.28%). Furthermore, age (P=0.006), gender (P=0.039), diabetes (P=0.003), history of infectious diseases (P=0.02), and duration of mask replacement (P<0.001) were important factors influencing the positive bacterial culture of the conjunctival microbiota. Although hypertensive patients exhibited a higher isolation rate of conjunctival bacteria, it did not reach statistical significance, and the history of cancer did not affect the isolation rate of the conjunctival microbial community in cataract patients before surgery.

• **CONCLUSION:** Potential changes are observed in the conjunctival microbiota among a sample of middle-aged and elderly individuals from Beijing, China. Notably, an increased isolation rate of *Corynebacterium* and *Micrococcus* is detected, suggesting a possible change in the microbial balance that requires further investigation and attention from the ophthalmological community. Advanced age, female gender, MGD, diabetes, a recent history of infectious diseases, and inadequate mask-wearing habits are potentially significant factors associated with the conjunctival microbiota. These factors should be considered in the development of strategies to prevent perioperative infections in cataract surgery patients.

• **KEYWORDS:** conjunctival microbiota; 16S rDNA; middle-aged and elderly population; mask; cataract surgery
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INTRODUCTION

The human body is a complex ecosystem. Research has shown that microbial communities exist in the human

gut, oral cavity, nasal mucosa, skin, and urogenital tract. Imbalances or transient increases in microbial communities may contribute to the development of certain diseases[1]. The conjunctiva is in direct contact with the environment, connected to the skin of the eyelids, and also harbors microbial colonization[2-4]. However, when there are changes in the ocular microenvironment or overall health status, these microorganisms may also contribute to infectious eye diseases such as blepharitis, conjunctivitis, and keratitis. Particularly, when the eyeball is compromised due to trauma or surgery, these microorganisms can become predisposing factors for infectious endophthalmitis[5]. Global epidemiological studies have indicated that the microbial community in the conjunctival sac is influenced by various factors such as ethnicity, environment, and geography, resulting in differences in composition, quantity, and antibiotic resistance[6-7].

Since the outbreak of COVID-19 in 2019, masks have played a crucial role in reducing virus transmission, and individuals have been conscientiously wearing masks in public settings. However, growing evidence suggests that during the pandemic, there has been an increasing incidence of eye discomfort, such as redness, tearing, and dryness[8]. Therefore, it is necessary to understand the current distribution of the conjunctival microbiota in individuals. In this study, we employed 16S rDNA gene sequencing to isolate and identify the conjunctival microbiota in cataract patients at the present stage. Additionally, we analyzed the potential factors that may influence the conjunctival microbiota.

PARTICIPANTS AND METHODS

Ethics Approval All participating individuals provided written informed consent according to the tenets of the Declaration of Helsinki. The Medical Ethics Committee of Peking University Third Hospital (Beijing, China) approved all procedures of the present study, including the procedure of accessing the clinical/personal patient data used in our research (approval number: IRB00006761-M2022841).

Participants From April 2022 to July 2022, 216 patients (216 eyes) who underwent cataract phacoemulsification and intraocular lens implantation in the Third Hospital of Peking University were selected. Randomly select one eye from each participant for inclusion in the study. The inclusion criteria were as follows: 1) age 45–90y, male or female, randomly enrolled in simple eye; 2) patients proposed for cataract surgery; 3) patients can understand the purpose of the trial, voluntarily participate and sign the informed consent form by themselves. The exclusion criteria were as follows: 1) suffer from blepharitis, conjunctivitis, keratitis, eyelid adenitis, *etc*. within 3mo; 2) previous ocular trauma; 3) history of eye surgery (excluding iris, fundus laser, conjunctival flaccid excision and pterygium excision for more than half a year); 4) antibiotics and immunosuppressants were used locally or systemically within 3mo; 5) non steroidal anti-inflammatory drugs have been used in the eyes within 3mo; 6) contact lens have been used within 3mo.

Clinical Factors Clinical factors from 216 patients were collected and used for analysis, including gender, age, history of meibomian gland dysfunction (MGD), history of hypertension, history of diabetes, history of cancer, history of infectious diseases, mask wearing habits, and blood tests for syphilis, hepatitis B, hepatitis C, and human immunodeficiency virus before cataract surgery. History of hypertension and diabetes was defined as the use of antihypertensive medication and diabetes medication and/or insulin therapy, respectively, prior to conjunctival culture. History of cancer did not include a history of benign tumors. History of infectious diseases was defined as the occurrence of local tissue or systemic inflammatory reactions caused by pathogens within the past year. Additionally, slit lamp examination and Keratograph 5M (Oculus Optikgeräte GmbH, Wetzlar, Germany) were used to evaluate whether patients had MGD. All examinations were conducted and the data were collected by two doctors (Sun YJ and Zhao TY).

Sample Collection and Bacteriologic Examinations To ensure the reliability of this study, conjunctival sac samples were collected by the same ophthalmologist. The samples were taken in a clean ophthalmic treatment room that had been disinfected with ultraviolet radiation. The physician was wearing a mask and sterile gloves while collecting the specimen. Sterile transport swabs are used to collect specimens. Samples were collected in the following order: 1) disinfect the skin around the subject's eyes with iodophor and instruct the patient to gaze upward; 2) exposure of the underlying bulbar conjunctiva and inferior fornix conjunctiva by turning the lower lid with a sterile cotton swab; 3) using the sterile transport swab, gently swab the lower conjunctival sac and conjunctival surface of the lower lid twice, starting from the inner canthus and rotating from inside to outside. Take care to avoid contact with the eyelashes and lid margin during the swabbing process.

Strain Isolation and 16S rDNA Gene Sequencing After collecting the conjunctival sac samples, the samples were stored at 4°C and the bacterial strains were isolated within 2h. The samples were placed on agar plates containing 10 g trypsin, 3 g ground beef, 5 g NaCl, 50 mL defibrinated sheep blood and 15 g agar in 1 L of medium. After incubation at 37°C for 24–72h, colonies with different phenotypes were selected for further analysis. Bacterial morphology was observed using a light microscope (Olympus Corporation, Tokyo, Japan). Genomic DNA was extracted from the isolated strains and the 16S rDNA gene was amplified using the method described

previously[9]. The polymerase chain reaction (PCR) products were purified and sequenced using an ABI3730 Genetic Analyzer (Beijing Genome Institute, China). After assessing the chimera formation of the product using the Bellerophon server, and comparing the 16S rDNA gene sequences with the GenBank database to search for relevant sequences using the BLAST program[10-11].

Cataract Surgery The surgeries were performed by the same experienced ophthalmologist using the same phacoemulsifier (Infinite, Alcon, USA). Patients were given 0.5% levofloxacin eye drops 4 times a day for 3d prior to surgery. Surface anesthesia with 0.4% oxybuprocaine hydrochloride eye drops was used. The surgical eye and surrounding attachments were disinfected with 0.5% povidone-iodine. Surface anesthesia with 0.4% oxybuprocaine hydrochloride eye drops was used. The surgical eye and surrounding attachments were disinfected with 0.5% povidone iodine. A 3-mm clear corneal incision was used for all surgeries. No intravenous antibiotics were administered during the surgery. At the end of surgery, tobramycin hyposemicarbazone ophthalmic ointment was applied to the ocular surface. Postoperatively, 0.5% levofloxacin, prednisolone acetate, and pralofene eye drops were used 4 times daily for 2wk.

Statistical Analysis SPSS 27.0 statistical software was used for data analysis, and normally distributed data were expressed as mean±standard deviation. Univariate analysis was performed by Chi-square test or Mann-Whitney *U* test to compare the incidence of each clinical factor in the two groups (patients with positive conjunctival sac cultures and patients with negative cultures), followed by multivariate analysis by multiple regression analysis. $P<0.05$ was considered a statistically significant difference.

RESULTS

Basic Characteristics This study included a total of 216 patients (216 eyes) who met the criteria. Among them, there were 92 men (92 eyes) and 124 women (124 eyes). The age range from 47 to 90y (mean±standard deviation: 68.55±8.59y). No cases of endophthalmitis were reported among the patients involved in this study following cataract surgery.

Bacterial Isolates and Rates Out of the 216 eyes examined, 78 eyes (36.11%) yielded bacteria. Among the total of 78 eyes, a single isolate was detected in 52 eyes (66.67%), while there were two isolated in 10 eyes (12.82%), three isolated in 14 eyes (17.95%), and four isolated in 2 eyes (2.56%).

Bacterial isolates and rates were shown in Table 1. In more detail, there were 60 strains of gram-positive rods were isolated, with 52 strains belonging to the genus *Cornebacterium*, accounting for 42.62% of all strains. Among them, there were 45 strains (36.89%) of *Corynebacterium macginleyi*, which was the most abundant strain among the isolated genus *Cornebacterium*. Other isolated gram-positive rods included 4 strains (3.28%) of *Bacillus*, 3 strains (2.46%) of *Arthrobacter*, and 1 strain (0.82%) of *Dermabacter jinjuensis*. A total of 55 strains (45.08%) of gram-positive cocci were isolated, including 38 strains (31.15%) of *Staphylococcus*, 12 strains (9.84%) of *Micrococcus*, 2 isolates (1.64%) each of *Enterococcus faecalis* and *Streptococcus,* and 1 strain (0.82%) of *Kocuria*. Among the *Staphylococcus spp.*, *Staphylococcus epidermidis* had the highest isolation rate with 27 strains. In addition, there were 6 gram-negative rods isolates (4.92%), including 5 strains (4.10%) of *Acinetobacter* and 1 strain (0.82%) of *Moraxella osloensis*. Only one type of fungus has been isolated, which is 1 strain (0.82%) of *Naganishia Diffluens*.

Table 1 Bacterial isolates and rates

Classification	*n* (%)
Gram-positive rods	
Corynebacterium spp.	52 (42.62)
Corynebacterium macginleyi	45
Corynebacterium accolens	3
Corynebacterium simulans	4
Bacillus spp.	4 (3.28)
Bacillus licheniformis	1
Bacillus velezensis	1
Bacillus subtilis	2
Arthrobacter spp.	3 (2.46)
Dermabacter jinjuensis	1 (0.82)
Gram-positive cocci	
Staphylococcus spp.	38 (31.15)
Staphylococcus epidermidis	27
Staphylococcus aureus	6
Staphylococcus capitis	5
Micrococcus spp.	12 (9.84)
Micrococcus luteus	7
Micrococcus antarcticus	5
Enterococcus faecalis	2 (1.64)
Streptococcus spp.	2 (1.64)
Kocuria spp.	1 (0.82)
Gram-negative rods	
Acinetobacter spp.	5 (4.10)
Acinetobacter haemolyticus	2
*Acinetobacter bouvet*ii	2
Acinetobacter johnsonii	1
Moraxella osloensis	1 (0.82)
Fungus	
Naganishia diffluens	1 (0.82)
Total	122

Association with Clinical Factors There is a strong correlation ($P=0.006$; Table 2) between age and the isolation rate of bacteria in the conjunctival sac of patients. It can be observed that the older the patients, the higher the bacterial isolation rate in the conjunctival sac.

Table 2 Bacterial detection rate dependent on age

Age (y)	Culture positive/total (%)
≤60	8/34 (23.53)
61–70	30/90 (33.33)
71–80	26/72 (36.11)
≥81	14/20 (70.00)

Chi-square test.

Table 3 Bacterial detection rate dependent on clinical factors *n* (%)

Clinical factors	Culture-positive patients (*n*=78)	Culture-negative patients (*n*=138)	Univariate *P*
Sex			0.039[a]
Male	26 (33.33)	66 (47.83)	
Female	52 (66.66)	72 (52.17)	
MGD			0.012[a]
Yes	50 (64.10)	64 (46.38)	
No	28 (35.90)	74 (53.62)	
Hypertension			0.608
Yes	48 (61.54)	80 (57.97)	
No	30 (38.46)	58 (42.03)	
Diabetes mellitus			0.003[a]
Yes	32 (41.03)	30 (21.74)	
No	46 (58.97)	108 (78.26)	
History of cancer			0.102
Yes	3 (3.85)	1 (0.72)	
No	75 (96.15)	137 (99.28)	
Screening for infectious diseases			0.020[a]
Yes	6 (7.69)	2 (1.45)	
No	72 (92.31)	136 (98.55)	
Mask replacement time			<0.001[b]
Yes	53 (67.95)	58 (42.03)	
No	25 (32.05)	80 (57.97)	

Chi-square test. MGD: Meibomian gland dysfunction. [a]*P*<0.05; [b]*P*<0.01.

Bacterial detection rate dependent on clinical factors was shown in Table 3.

In 78 cases of isolated bacterial strains from the eyes, the bacterial isolation rate was higher in female patients compared to male patients, with a twofold difference that is statistically significant (*P*=0.039). The bacterial isolation rate in patients with MGD was significantly higher than in non-MGD patients (*P*=0.012). Diabetic patients had a significantly increased bacterial isolation rate compared to non-diabetic patients, with a statistically significant difference (*P*=0.003). Patients with a recent history of infectious diseases had a significantly higher bacterial isolation rate than patients without such history, with a statistically significant difference (*P*=0.02). Additionally, in terms of mask replacement time, patients who did not have the habit of replacing their masks every 4h had a significantly higher bacterial isolation rate than patients who had this habit (*P*<0.001). Although the bacterial isolation rate was higher in patients with hypertension, it was not statistically significant. The history of tumors also did not affect the preoperative bacterial isolation rate in patients with cataracts.

DISCUSSION

Infectious endophthalmitis is one of the most serious complications of ocular surgery. Therefore, preventing post-cataract surgery endophthalmitis is a matter of great concern for ophthalmologists. The pathogens causing infectious endophthalmitis primarily originate from the ocular surface, such as bacteria from the conjunctival sac, eyelids, and meibomian glands[12-13]. These microorganisms can be reduced through disinfection methods. However, even with the most rigorous disinfection protocols currently available, it is not possible to completely eliminate them[14]. Adequate knowledge of the characteristics of the bacterial microbiota in the conjunctival sac in patients is crucial for us to improve measures to prevent postoperative endophthalmitis. In this study, we investigated the composition of conjunctival bacteria in recent middle-aged and elderly patients and analyzed the

systemic clinical factors associated with positive conjunctival bacterial cultures.

In recent years, an increasing number of studies have shown that the conjunctival sac of normal individuals harbors a microbiota. These bacteria coexist within the conjunctival sac, maintaining a dynamic equilibrium and inhibiting the growth and invasion of pathogenic bacteria[3]. *Staphylococcus epidermidis*, *Streptococcus spp.*, *Staphylococcus aureus*, *Propionibacterium acnes*, *Corynebacterium spp.*, *Streptococcus spp.*, and *Haemophilus influenzae* are the most commonly isolated microorganisms in the normal conjunctival microbiota. Among them, earlier studies found that *Staphylococcus aureus* had the highest isolation rate among numerous studies. Additionally, anaerobic bacteria and fungi may also be occasionally isolated[13,15-16].

The bacterial isolation rate in this study was 36.11%. The detection rate is consistent with literature report[3,17]. A total of 122 strains were detected in the conjunctival sac of 78 patients with positive bacterial culture. A total of 60 gram-positive rods were detected, accounting for 49.18% of the total, while 55 gram-positive cocci were detected, constituting 45.08%. Gram-negative bacteria were detected in 6 samples, comprising 4.92% of the total, whereas only 1 fungal species was identified, representing 0.82%. This study revealed that the most abundant genera in the conjunctival sac were *Corynebacterium* (52%), *Staphylococcus* (38%), *Micrococcus* (9.84%), *Acinetobacter* (4.10%), and *Bacillus* (3.28%). There are still some discrepancies between our research findings and previous reports. These differences may be associated with variations in sample collection methods, detection techniques as well as the living environment, lifestyle, and physiological factors of the study participants.

As the gold standard for microbial detection, clinical laboratories commonly employ culture-based methods to identify samples at the species level and measure bacterial density. However, the results are often influenced by factors such as culture conditions and incubation time. In this study, 16S rDNA gene sequencing was utilized, which has the capability to identify unculturable microorganisms, providing an efficient, comprehensive, and accurate research approach for microbial community composition[9]. The study by Zhou *et al*[18] revealed that *Corynebacterium*, *Streptococcus*, *Propionibacterium*, *Bacillus*, and *Staphylococcus* were the top five abundant genera in the healthy conjunctival sac. Huang *et al*[4] found that the most abundant genera in the normal conjunctival sac were *Corynebacterium* (28.22%), *Pseudomonas* (26.75%), *Staphylococcus* (5.28%), *Acinetobacter* (4.74%), and *Streptococcus* (2.85%). They also employed the method of 16S rDNA gene sequencing, and their findings were similar to ours. These collective research findings suggest that the isolation rate of *Corynebacterium* in the conjunctival sac may be gradually increasing in recent years, possibly surpassing *Staphylococcus* as the most abundant genus in the conjunctival sac. As one of the "core resident microbial communities" on the ocular surface, *Corynebacterium* has the function of enhancing eye immune balance and host defense. Studies have shown that this genus can induce γδT cells in the ocular mucosa to produce commensal-specific interleukin-17. This response is central to local immunity, as it promotes the recruitment of neutrophils and the release of antibiotics in tears, protecting the eyes from pathogenic bacterial infections[19]. Ge *et al*'s study[20] confirmed that the decrease in the abundance of *Corynebacterium* is associated with fungal keratitis.

In this study, the genus *Micrococcus* also caught our attention, with a significantly increased isolation rate compared to previous studies. *Micrococcus* is commonly found in the skin, soil, and can also be isolated from food and air. It is considered an opportunistic pathogen, capable of causing local tissue infections such as wounds, but can also lead to severe infections such as endocarditis and brain abscesses[21-22]. An *et al*[23] utilized 16S rDNA sequencing to examine the conjunctival microbial community in 18 healthy adults. They found a relatively high isolation rate of the genus *Micrococcus* (22.2%), ranking third after *Staphylococcus* and *Corynebacterium*. This finding aligns with our results. Wang *et al*[24] found that after three days of preoperative use of levofloxacin eye drops, *Staphylococcus epidermidis*, *Kocuria rosea*, and *Micrococcus luteus* were the top three strains with the highest positive culture rates. This suggests that preoperative use of antibiotics does not completely eliminate the presence of the *Micrococcus* in the conjunctival sac, which may result in opportunistic eye infections after surgery. The increased isolation rate of the *Micrococcus* in the conjunctival sac should raise concerns among ophthalmologists and prompt further exploration of safer and more effective preoperative disinfection strategies.

This study found that the isolation rate of bacteria in the conjunctival sac showed an increasing trend with age. The population aged 80y and above had the highest bacterial isolation rate (70.0%). In other words, elderly patients are at a higher risk of positive conjunctival sac cultures before cataract surgery. Our study also demonstrated that certain clinical factors, such as being female, having MGD, diabetes, and recent history of infections, were associated with a higher bacterial load in the conjunctival sac. This has been confirmed in previous studies as well[2-3,24].

Due to the ongoing COVID-19 period, wearing masks has become a necessity in hospitals and public places. Another important finding in our study was that individuals who did

not have the habit of regularly changing their masks had a significantly higher isolation rate of bacteria in the conjunctival sac compared to those who had this habit. Therefore, we speculate that mask-wearing habits can influence the microbial composition in the conjunctival sac. Prolonged mask-wearing indeed increases the likelihood of ocular irritation and discomfort symptoms, leading to more ocular surface diseases such as dry eye and corneal epithelial damage[8]. Previous studies have found that the microbial subgroups on both the inner and outer surfaces of masks can lead to changes in facial and gut microbiota[25-26]. During the pandemic, there was a significant accumulation of *Lactobacilli* and *Corynebacterium* in the gut, and the abundance of *Bacteroides* gradually increased, resulting in significant differences in bacterial species before and during the outbreak. This study also found a significant increase in the isolation rate of *Corynebacterium* in the conjunctival sac of the current population compared to before. Additionally, facial microbiota diversity decreased[27], but the pathogenicity (functionality) of facial microbiota significantly increased, leading to more skin issues. Alterations in gut microbiota (ecological disruption) can contribute to various eye diseases[28] such as uveitis and dry eye disease. Since there exists a gut-eye or gut-eye-lacrimal gland microbiota axis, disruptions in gut microbiota may also lead to changes in ocular surface microbiota[29].

In addition to the aforementioned factors, the impact of masks on the microbial composition in the conjunctival sac may also involve the following factors. First, during mask-wearing, exhalation generates an upward airflow[8]. This upward airflow disrupts the lipid layer of the tear film, accelerating the evaporation of the ocular surface tear film. As tears serve as a natural barrier on the ocular surface, the disruption of this barrier makes it easier for bacteria to adhere. Moreover, tears contain abundant antimicrobial substances such as lysozyme, cationic antimicrobial peptides, and surfactant protein D[30]. When tear film stability decreases, the antimicrobial abilities of these molecules may be compromised, leading to changes in the bacterial population in the conjunctival sac. Second, the upward airflow contains a high concentration of carbon dioxide (4%–5% in exhaled gas) compared to inhaled air (0.4%), which can lead to decreased corneal nerve sensitivity[31]. Additionally, the upward airflow generated during exhalation can lead to changes in the temperature around the eyes. Kapelushnik *et al*[32] investigated the changes in ocular surface temperature during each respiratory cycle while wearing surgical masks and observed a significant increase of approximately 0.5°C, particularly at the eyelid margin. This temperature elevation persists over a prolonged duration, leading to a decrease in tear film stability, barrier disruption, and increased susceptibility to bacterial colonization. Although there is no direct research on the impact of temperature on ocular surface microbiota, Sepulveda and Moeller[33] found that temperature alterations increased gut microbiota diversity in animals. This suggests that ocular microbiota may also adapt to specific temperature conditions, and temperature changes may affect the adaptability of resident microbiota to the host, leading to disruption of the existing microbiome and the emergence of new resident microbial populations.

There are limitations to this study. First, our sample size was still limited, and individuals' microbial compositions can vary significantly. More samples from larger regions, different seasons, and various age groups are needed. Second, the influence of occupational exposure and living environment on the conjunctival sac microbiota was not further investigated. Third, the differences between microbial compositions inside masks and in the conjunctival sac were not examined. Finally, further research is needed to explore the drug sensitivity characteristics of the conjunctival sac microbiota at the current stage, providing insights for optimizing clinical preoperative antimicrobial strategies.

In conclusion, we employed 16S rDNA gene sequencing to isolate and identify the microbial community in the conjunctival sac of cataract patients who had been wearing masks for nearly two years prior to surgery. The results revealed that the most abundant microbial genera in the conjunctival sac were *Corynebacterium*, *Staphylococcus*, *Micrococcus*, *Acinetobacter*, and *Bacillus*. Specifically, the isolation rate of *Corynebacterium* and *Micrococcus* showed an increase. Furthermore, the isolation rate of conjunctival bacteria was closely associated with age, gender, MGD, diabetes, recent history of infectious diseases, and mask-wearing habits. The changes in the composition of the conjunctival sac microbiota may have implications for ocular health and thus warrant attention from ophthalmologists. Further research is essential to optimize preoperative antibacterial strategies for clinical application.

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