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Molecular Analysis of the Ocular Microbiome Dominant Bacteria in Conjunctival Squamous Cell Carcinoma Patients

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ABSTRACT

Background and Aim: Conjunctival squamous cell carcinoma (CSCC) is the final stage of ocular surface squamous neoplasia (OSSN). The microbiome plays a critical role in eye disease and health. Changes in the eye microbiome may be involved in the development of OSSN.

Materials and Methods: The sampling for this case-control study involved rolling sterile rayon-tipped swabs on the conjunctival tissue of both patients and controls. The detection of bacterial communities, including *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Staphylococcus haemolyticus* (*S. haemolyticus*), *Corynebacterium* spp., *and Acinetobacter* spp. The study was conducted using the TaqMan quantitative real-time polymerase chain reaction (qPCR) method, which targets the 16S rDNA gene from bacteria in various samples.

Results: The results showed that the frequency of *Corynebacterium* spp. was the same in both the patient and control groups (77.88%). Further, it exhibited the highest copy number compared to other bacteria. In general, a significant difference was observed in the total amounts of *P.aeruginosa*, *S. aureus*, and *S. haemolyticus* among patients, with a P-value < 0.001, additionally, *Acinetobacter* spp. showed a significant difference with a P-value = 0.004.
88.9% of *S. haemolyticus* (*P*=0.002) and 83.3% of *P. aeruginosa* (*P*=0.033) were detected in the OSSN group, and no detection of these bacteria was observed in any of the control samples.

Conclusion: The study found a significant difference in some bacterial strains between patients with OSSN and those without OSSN. Understanding the function and composition of the ocular surface microbiome can provide insights into ocular surface diseases.

Keywords: Ocular Microbiome, Eye Microbiota, Ocular Surface Squamous Neoplasia, Conjunctival Squamous Cell Carcinoma, Real-time qPCR

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1. Introduction

Ocular Surface Squamous Neoplasia (OSSN) is a neoplastic process that affects the conjunctiva and encompasses a wide range of conjunctival malignancies, including mild epithelial dysplasia and

conjunctival squamous cell carcinoma (CSCC) (1). The causes of OSSN include human immunodeficiency virus (HIV) infection, exposure to ultraviolet B radiation, allergies, smoking, genetic factors, and human papillomavirus (HPV) types 16 and 18 (2, 3). It reported an annual frequency of 0.5 per million in the United Kingdom (UK) (4). The incidence rates of OSSN vary from 0.01 to 3.4 per 100,000 individuals per year (5). Unfortunately, the optimal treatment for patients with this disease involves surgery, tumor removal, and topical chemotherapy. Although the rate of tumor recurrence is high and requires conservative treatment methods (1, 6).

The massive Human Microbiome Project (HMP) began in 2008 with the primary goal of identifying microorganisms from five different organs in the body, including the nasal passages, skin, oral cavity, urogenital tract, and gastrointestinal tract (7). Dysbiosis or alterations in microbial composition, can occur due to dietary changes, antibiotic exposure, or infection (8). Although the eyes are crucial, the HMP project did not investigate the eye microbiome due to the low microbial abundance on the surface of the eye (9). Ocular surface microbiome (OSM) composition is influenced by environmental, host, and iatrogenic factors. OSM dysbiosis can be related to various systemic disorders (10). The surface of the eye has its own bacterial microbiome, which includes both opportunistic organisms and probiotics. By creating opportunities such as eye trauma, opportunistic pathogens in the eye microbiome may cause infections (11). One of the key functions of the eye microbiome is to maintain the balance of microorganisms on the surface of the eye. A healthy ocular microbiome helps protect the eye from pathogens by competing for resources and producing antimicrobial substances that can inhibit the growth of harmful bacteria and viruses (10, 12). The eye surface microbiome plays a crucial role in maintaining local homeostasis and preventing the growth of pathogenic species. Many authors have suggested that changes in the eye microbiome are associated with various diseases (13).

Studies that have characterized the eye microbiome using culture-independent methods have shown greater microbial diversity compared to studies that rely on culture-based assessments. The dominant phyla found on the surface of the eye are Firmicutes, Proteobacteria, and Actinobacteria (14, 15). Various studies have utilized culture-independent techniques to differentiate between microbial communities at the conjunctival level. Most of the studies *on Corynebacterium* focused on the visible surface and reported lower frequencies *of Staphylococcus*, *Streptococcus*, *Acinetobacter,* and *Pseudomonas (16-18)*. 16S sequencing revealed major microbial species including *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., *Propionibacterium* spp., and *Haemophilus* spp. (19). According to numerous studies, the precise correlation between ocular surface stimulation and OSM remains unclear (7). One of the most common bacteria associated with eye infections is *Staphylococcus aureus* (*S. aureus*). This gram-positive bacterium is part of the natural flora of the skin and mucous membranes, including the eyes. However, under certain conditions, it can invade the eye and cause an infection in the eye, leading to diseases such as conjunctivitis, endophthalmitis, and keratitis (20). Another important bacterium involved in eye diseases is *Pseudomonas aeruginosa* (*P. aeruginosa*). This gram-negative bacterium is known for its ability to grow in moist environments and is especially dangerous for those who wear contact lenses. *Pseudomonas* infection can lead to corneal ulcers, which are painful and can cause permanent damage if left untreated (21). Through the application of advanced sequencing techniques and analysis of the composition and diversity of the bacterial flora, researchers have determined that *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, and *Acinetobacter*, are the main components found on the surface of the eye (22). It is still unclear whether OSM is involved in the development of these infections or whether these diseases are affected by OSM (23). Based on research findings, molecular methods for detecting microbes in severe diseases are promising.

For this study, we will examine the eye microbiome of both healthy individuals and patients. We will analyse the conjunctiva of the eye using TaqMan quantitative real-time polymerase chain reaction (qPCR) to determine the abundance and composition of the main bacteria associated with OSSN in the studied groups. This research in Iran is the first to examine the relationship between the eye microbiome and a specific disease. Since there are no existing reports in Iran, we have decided to investigate the microbial composition of patients with OSSN. In this study, according to previous studies, we selected a number of bacteria that include *P. aeruginosa* , *S. aureus*, *Staphylococcus haemolyticus* (*S. haemolyticus*), *Corynebacterium* SPP., and *Acinetobacter* SPP. By understanding the composition and diversity of these bacterial communities, we can gain valuable insights into their potential roles in eye health and disease.

2. Materials and Methods

Patients and Samples

Here, we studied a total of 36 cases, including 18 cases with OSSN and 18 cases with healthy individuals.

These cases were collected from Rasoul Akram Hospital in Iran over a period of 18 months, from June 2018 to November 2020. The Ethics Committee of Iran University of Medical Sciences approved the study protocol, and all participants provided written informed consent. The presence of OSSN was confirmed through clinical examination, and impression cytology, and final approval was obtained through biopsy. We recorded pertinent background information, such as age, gender, living environment, previous medical history, ocular history, antibiotic usage, and surgical history. We exclude patients with any signs of ocular surface irritation or illness, persistent eye contamination, contact lens use, or administration of topical or systemic medications within the previous 6 weeks.

Sample Collection

To begin the analysis, we collected conjunctival swabs. Sampling was performed during surgery by gently rolling sterile rayon-tipped swabs on the conjunctival tissue of both patients and controls. Immediately after collecting the sample, the swabs were placed in tubes containing 1.5 mL of phosphate-buffered saline (PBS) without coming into contact with the skin to prevent contamination. The swabs were immediately transported to the laboratory on ice, and DNA extraction was completed within less than 6 hours.

Primer and Probe Design

To avoid false positive results, all TaqMan probes and primers used to identify each bacterium were designed with high precision for the first time in this study. For this purpose, bacterial 16S rRNA sequences were obtained using the SILVA high-quality ribosomal RNA database, and then the sequences were converted to 16S rDNA. Also, the specificity of the designed primers and probes was confirmed using AlleleID and Primer BLAST software (24, 25). The specific sequences of TaqMan probes and primers are in Table 1.

Table 1. 16S rDNA gene-targeted specific primers and TaqMan probes

Target Bacteria	Primer/ Probe *	Oligonucleotide sequence (5´-3´)	Size (bp)	Product size (bp)	Ref.
***Corynebacterium* spp.**	Primer F	AACTTGAGTGCTGTAGGGGTRA	22	115	This study
	Primer R	CTCCTCAGCGTCAGTWACTGC	21		
	Probe	AGCGTCAGTTACTGCCCAGAG	21		
P.aeruginosa	Primer F	GTGGTTCAGCAAGTTGGATGTG	22	190	This study
	Primer R	CCACGCTTTCGCACCTCAG	19		
	Probe	CGCCTTCGCCACTGGTGTTCCTTCCTATA	29		
S. aureus	Primer F	AAGTCGAGCGAACGGACGAG	20	250	This study
	Primer R	CTCTCAGGTCGGCTATGCATCG	22		
	Probe	ACCTTACCAACTAGCTAATGCAGCGCGGAT	30		
S.haemolyticus	Primer F	CCGTATTAGCTAGTTGGTAAGGTAAC	26	250	This study
	Primer R	GTACCGTCAAGACGTGCATAG	21		
	Probe	CCAAGGCGACGATACGTAGCCGACCT	26		
***Acinetobacter* spp.**	Primer F	GTKAGTGGACGTTACTCGCAG	21	150	This study
	Primer R	AGTTAAGCTCAGGGATTTCACATCC	25		
	Probe	CGCCTACGCACGCTTTACGCCCAGTA	26		

Primers F (forward), R (reverse), and probes targeting the 16S rDNA gene.

DNA Extraction

We extracted the total genomic DNA from the eye samples using the pure PCR template preparation kit (Favorgen, Taiwan, and Lot. No.BJB04119B05) based on the producer's rules. The DNA concentration was determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and confirmed by running agarose gel electrophoresis. The quality and concentration of DNA were checked using

1% agarose gel electrophoresis (Figure 1) and a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

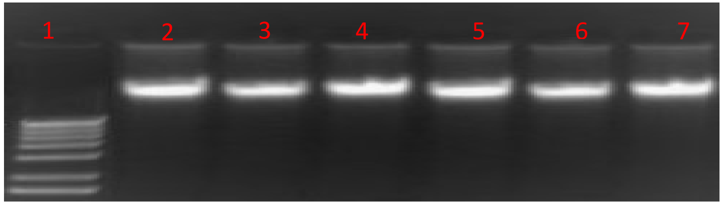

Figure 1. The image shows a gel electrophoresis run using a DNA ladder (100 bp) in column 1. This run was performed for the extraction of eye bacteria from the genome using a dedicated DNA extraction kit.

qPCR Quantification

The bacterial community response to differences among various species was measured using real-time TaqMan qPCR on Rotor-Gene 6000 real-time PCR cyclers (Qiagen Corbett, Hilden, Germany). This analysis employed group-specific probes and primers targeting the 16S rDNA. All qPCR tests were run in duplicates. Each qPCR test was performed in a total volume of 20 µl, which included 100 ng of extracted DNA and 9 µl of amplicon Master Mix for probe without ROX (SinaClon-Iran, CAT. No., PR901638), 0.5 µM of each primer, 0.25 µM of the probe. A real-time PCR test was performed using the Corbett Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science, Australia).

Every amplification protocol included a primary denaturation stage of 15 minutes at 95°C, followed by 40 cycles of 20 seconds at 95°C for denaturation, annealing/elongation for 45 seconds at 62°C, and a final elongation stage at 72°C for 20 seconds. For each analysis, negative controls were included that contained all elements except for the template DNA. The displayed information represents the average amount of duplicate real-time qPCR examinations. We obtained positive control strains used in this investigation from the American Type Culture Collection (ATCC). In this study, we used *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *S.haemolyticus* ATCC 29970, and *Acinetobacter* ATCC 51819 and *Corynebacterium* ATCC 21799 were used as quality control strains **(26)**.

We defined the parameter cycle threshold (Ct) as the point at which the probe binding to the double-stranded DNA produced the initial detectable fluorescence. With the serial dilution of genomic DNA from the standard strain, a standard curve was designed for each qPCR trial. Then, by utilizing a standard curve and defining the copy number concentration, we performed absolute quantification. As a result, we are able to report the quantification of the copy number of any bacteria per specimen **(18, 27)**.

Statistical Analysis

For data analysis, we utilized the two-sample t-test, Levene's test, and Mann-Whitney test on a subset of samples to assess quantitative differences between OSSN and control samples for each bacterium. GraphPad Prism version 8.3.0, Minitab version 16.2.0, and SPSS version 19.0 (SPSS Inc., Chicago, IL. USA) were used for statistical analysis. Statistical significance was determined for P-values below 0.05. We converted bacterial composition quantities into fold alteration quantities (Fold change = $2^{-\Delta\Delta Ct}$) before conducting the statistical analysis.

3. Results

Subjects

During an 18-month period, a total of 36 samples were collected from Rasoul Akram Hospital in Iran. Out of these samples, 18 samples of patients with OSSN and 18 samples of healthy people (without OSSN) were included. The differences in the characteristics of the participants in the control and case groups are shown in Table 2.

Quantitative PCR Analysis of Bacterial Groups

The present study used qPCR to analyse the diversity of bacterial communities in patients with OSSN compared to healthy controls. Overall, the analysis was based on changes in five bacteria, including *S. aureus*, *P. aeruginosa* , *S. haemolyticus*, *Corynebacterium* spp., and *Acinetobacter* spp. After removing the outliers, the results showed that the frequency of *Corynebacterium* SPP. was the same in both the patient and control groups (77.88%) and had the highest copy number compared to other bacteria. In general, a significant difference was observed in the total amounts of *P.aeruginosa*, *S. aureus*, and *S. haemolyticus* among patients, with a P-value < 0.001, additionally, *Acinetobacter* spp. showed a significant difference with a P-value=0.004. *S. haemolyticus* was

detected in 88.9% of the OSSN group (*P*=0.002) while *P. aeruginosa* was detected in 83.3% (*P*=0.033). No detection of these bacteria was observed in any of the control samples. *S. haemolyticus* and *P. aeruginosa* copy numbers were significantly higher in patients in the OSSN group compared to the control group, as shown in Figure 2.

Table 2. Characteristics of the study participants

Characteristics	Patient group	Control group	P-value
Number	18	18	-
Male	15 (78%)	10 (61%)	0.062
Female	3 (22%)	8 (39%)	0.062
Age (mean ± SD)	54.77± 15.10	55.33± 10.13	-
Smoking	6	2	0.142
Alcohol consumption	3	0	0.110
Doing sports activities	8	11	0.317
Sun exposure	11	3	0.006

Relationships between both groups were analysed using the independent T-test and chi-square test. The values show a significant difference when P is less than 0.05.

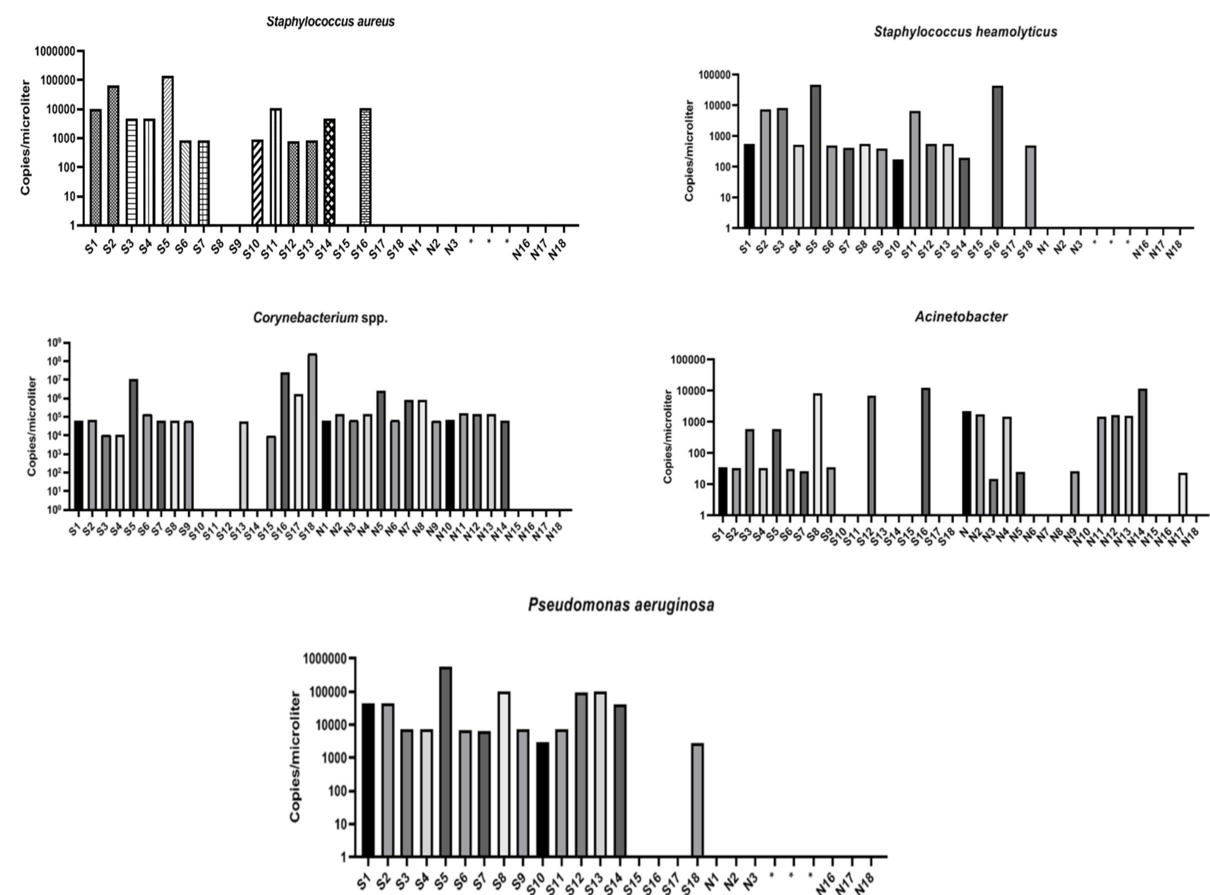

Figure 2. qPCR quantification of bacteria in the OSSN group and control group. Each bar represents one sample, either from the OSSN or control groups. The order of the samples is the same for each bacterium, with "S" indicating the OSSN group and "N" indicating the control sample.

4. Discussion

Recently, few studies have evaluated the relationship between changes in the ocular surface microbiome and ocular diseases **(28)**. A previous examination showed that OSM contained the same four frequent species found by culture: *Propionibacteria*, *Streptococcus*, *Staphylococcus*, and *Corynebacteria* **(18)**. In this study, we examined the *Corynebacterium* spp., *P. aeruginosa*, *S. aureus*, *S. haemolyticusm*, and *Acinetobacter* spp. Our results are consistent with a previous study by Graham *et al.* **(29)**, which demonstrated that the ocular conjunctival microbiome undergoes changes in patients with conditions like dry eye disease **(29)**. In our study there was a significant difference between healthy subjects and OSSN patients. *P. aeruginosa*, *S. aureus*, *S. haemolyticus* (P<0.001), and *Acinetobacter* spp. (P=0.004).

In another study, the presence of *S. aureus* colonies was shown in 27.78% (5/18) of vernal keratoconjunctivitis (VKC) samples, compared to 4.55% (1/22) in the control group (P=0.041). They indicated a possible relationship between *S. aureus* colonization and the occurrence of VKC. Similar to our study, the distribution of *S. aureus* was 77.8% (P<0.001) , which presented a significant difference in patients with OSSN **(30)**. About blepharitis, Lee, Oh **(31)** determined a shift in the microbial composition, with an increase in the prevalence of *Corynebacterium* spp., and *S. aureus* in comparison to healthy controls. Unlike our study, there was no significant change in the *Corynebacterium* spp. observed in both healthy individuals and patients with OSSN **(31)**. As observed in our study, the distribution of *P. aeruginosa* (with a frequency of 83.3%) (P<0.001) exhibited a significant difference in patients with OSSN. Some researchers have demonstrated the role of OSM in regulating the induction of infectious keratitis caused by *P. aeruginosa* **(32)**. In our study, it seems that the proliferation and survival of cancer cells can be caused by this and thus contribute to the development of OSSN. Interestingly, similar to our results, *S. aureus* (77.8%), and *S. haemolyticus* (88.9%) (P<0.001) showed a significant difference in patients with OSSN when analysed by qPCR. However, when examined using traditional culture techniques, it was found that the ocular surfaces of patients with Stevens-Johnson syndrome were more likely to grow CNS (Coagulase-negative *staphylococci*) and *S. aureus* compared to healthy controls **(33)**. These findings may be because certain strains of *S. aureus* produce a toxin called Pantone-Valentine Leukocidin (PVL), which has been shown to induce the expression of pro-inflammatory cytokines and chemokines in cells, thereby exacerbating the local inflammatory response **(34)**.

The remarkable finding in this study was the lack of a significant difference in the frequency of *Corynebacterium* between patients and the healthy control group. This result may be attributed to previous research indicating that *Corynebacterium* inhibits the Interleukins-17 (IL-17) response, which is induced by γδT cells in the ocular mucosa to fight fungal and bacterial infections on the ocular surface of mice **(35)**. Additionally, further research should investigate the pathways that these bacteria play in the development of OSSN. It is possible that bacteria in the conjunctiva may cause long-term inflammation, which is a known factor in the development of various types of cancer, including OSSN. It seems that bacteria release substances that cause inflammation, disrupt the normal function of conjunctival cells, and consequently lead to irregular growth of squamous cells, resulting in tumor formation **(36)**. The exact mechanisms by which these bacteria contribute to the development of OSSN are not yet fully understood , but it is possible that the presence of these bacteria on the surface of the eye causes an inflammatory response, leading to the release of various cytokines and chemokines. These inflammatory mediators can promote cellular proliferation and DNA damage, ultimately contributing to the development of neoplastic lesions **(37)**.

Moreover, the incidence rates of OSSN differ worldwide based on location, with a higher number of cases reported in the southern hemisphere **(38)**. It has been observed in studies that exposure to the sun is associated with OSSN **(39)**, and our data in Table 2 (P=0.006) also showed a significant association with this issue. Further, in the studies that were observed, OSSN was found to be predominant in men **(40)**. In our study, 78% of the patients were men. It seems that the variation in the findings of multiple studies may be attributed to differences in geographical area, disease severity, and disease type **(41)**. This is the first study in Iran that investigated the most important bacterial genera of the eye microbiome, particularly in patients with OSSN. These findings present new opportunities for therapeutic interventions that focus on the microbiome in patients with OSSN. Therefore, further studies are necessary to compare the data, confirm these findings, and understand how changes in bacterial communities contribute to the pathogenesis of OSSN.

5. Limitation

It should be noted that the study has a limitation due to the small number of participants. To obtain more accurate results, it is recommended that future research be conducted using metagenomic profiling

and advanced sequencing technology in a larger and more diverse community. Also, it is important to keep in mind that the study results from one center may not be applicable to other geographic locations. For this reason, it is recommended to conduct longitudinal research to gain a better understanding of the dynamics of the microbiome as the disease progresses **(18, 42)**.

6. Conclusion

The study of the ocular microbiome is crucial for enhancing our understanding of the diversity of bacterial communities in conjunctival squamous cells among Iranian patients with OSSN. The results of the present study suggest that differences in the composition and diversity of bacterial abundance may be related to this disease. The findings indicated that the frequency of *Corynebacterium* spp. was equal in both the patient and control groups. Patients showed a significant difference in the total amounts of *P. aeruginosa*, *S. aureus*, *S. haemolyticus*, and *Acinetobacter* spp. This finding may be attributed to differences in geographic region, severity, and type of disease. Also, the presence of some bacteria on the surface of the eye may cause an inflammatory response, leading to the release of immune system mediators and the progression of the disease. Strategies aimed at modulating the conjunctival bacterial community, such as probiotics or targeted antibiotics, could potentially help reduce inflammation, inhibit tumor growth, and improve the overall prognosis for patients with OSSN. By understanding the composition and functional potential of these bacteria, we can further investigate their roles in maintaining ocular health and understanding ocular diseases. This knowledge may lead to the development of targeted interventions to manipulate the eye microbiome and enhance eye health outcomes. Continued research in this field will undoubtedly shed more light on the complex relationship between bacteria and OSSN, ultimately leading to novel therapeutic strategies.

Acknowledgment

Not applicable.

Ethical Statement

This investigation was performed in accordance with the ethical guidelines as formerly confirmed by the Iran University of Medical Science, Tehran, Iran (project no: “IR.IUMS. FMD. REC.1397.314”).

Conflict of Interest

The authors have stated that there are no conflicts of interest in the study

Authors’ Contribution

The work was conceived and designed by SR, NM, and HA. General research was conducted by ST and AD. Samples were collected by SR, NM, and HA. ST conducted microbiological work on the samples and wrote the manuscript. Statistical analyses were carried out by NB and ZC. The manuscript was edited by ZC, AD, and SR. It is important to note that all authors have approved the final version of the manuscript.

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