Iranian Journal of Microbiology
Volume 16 Number 4 (August 2024) 576-583

ORIGINAL ARTICLE

Investigation of eye flora in cats infected with Herpesvirus and Calicivirus

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Received: July 2023**, Accepted:** March 2024

ABSTRACT

Background and Objectives: The ocular surface is perpetually exposed to the external environment, rendering it susceptible to microbial contamination. The ocular surface microbiota consists of non-pathogenic microorganisms that inhabit the conjunctiva and cornea. This study's objective was to extensively review the prevalence of bacterial and fungal organisms in the conjunctiva of healthy and diseased cats. (Herpes- and Calici-infected groups).

Materials and Methods: The current study was performed on 240 cats that had visited veterinary health centers (Tehran, Iran) for examination. Sterile swabs from each cat's eyes were investigated for microbiological assessment. After sample collection, viral pathogens (Herpes and Calici viruses) were isolated and identified using the PCR method. The ages of the investigated group were 3.76, 3.93, and 4.15 months.

Results: The highest frequency of bacteria in the normal, Herpes-infected/Calici-infected, and Herpes/Calici-infected groups were associated with *Staphylococcus intermedius* and *Streptococcus agalactiae*, *Staphylococcus epidermidis*, and *Staphylococcus intermedius*, respectively. In addition, it was found that the high prevalence of fungal microorganisms in the isolated samples was related to yeasts, *Aspergillus (Aspergillus fumigatus, Aspergillus niger)*, and *Penicillium* species.

Conclusion: Bacterial prevalence was significantly higher in all groups than the prevalence of fungi in the eyes of cats. The statistical comparison between the study groups regarding microbial and fungal frequency showed that significant differences were found between them, such that the frequency was higher in all disease groups, against the control group. In addition, a significant relation was observed between the Herpes-infected and Calici-infected groups regarding microbial and fungal prevalence.

Keywords: Ocular surface; Cat; Herpesvirus; Calicivirus; Microbiota

INTRODUCTION

Eyes are shielded by the eyelids and other supportive structures. Its internal makeup comprises two distinct compartments. The first encompasses the anterior and posterior chambers, iris, lens, vitreous body, retina, ciliary body, choroid, and intrinsic eye muscles. The second compartment, external to the first, consists of the conjunctiva, cornea, sclera, and tear film (1). The internal compartment of the eye is physically isolated from the immune system by the eye's blood-retinal barrier. It is kept free from any germs, ensuring a sterile environment (2). On the other hand, the outer compartment of the eye is exposed to the

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external environment and can easily become contaminated with harmful microorganisms (3-5).

The eye employs several defense mechanisms to ward off harmful microorganisms. These include a tear film rich in antimicrobial substances that inhibit pathogen growth, the conjunctiva acting as a physical and biological shield, an innate immune system in the ocular surface epithelium capable of recognizing potential pathogens, and a resident population of harmless microbes that help prevent colonization by harmful ones (6-8).

Multiple investigations have established the presence of commensal bacteria on the surface of the eye (9, 10). Healthy individuals have a natural microbiome on the surface of their eyes, which consists of both virus and bacterial communities (11). The presence of ocular microbiota is generally recognized. Multiple studies have demonstrated a significant intricacy in the makeup of the ocular microbiome (9, 12).

As mentioned, the ocular surface is comprised of the corneal epithelium and the highly exposed mucous membrane of the body, known as the conjunctiva. Moreover, it is a predominantly open system that is constantly exposed to a wide range of pathogenic and non-pathogenic microorganisms due to its continuous contact with the environment. This approach utilizes the body's adaptive and innate immune systems to inhibit the growth of harmful pathogens on the surface of the eye (13). Nevertheless, it is probable that there exists a factor of harmonic interaction among microbiota, functioning in a mutually beneficial and mutually dependent manner to prevent excessive development or establishment of harmful germs. The phenomenon of mucosal tolerance enables the ocular surface bacteria to coexist in its surroundings without causing any immune response. Cats can get infectious conjunctivitis, which is commonly caused by either viruses or bacteria. This condition is a common source of eye issues in veterinary medicine, where both harmful and opportunistic bacterial germs play a role in the development of the disease. Topical ocular medication, such as erythromycin, are the recommended treatment for feline conjunctivitis and keratitis. Antibiotics can have a detrimental effect on the microbial population of the surface of the eye, which could potentially lead to the invasion of harmful species and the development of eye diseases (13).

Feline herpesvirus type 1 (FHV-1), which is responsible for causing feline viral rhinotracheitis, is highly prevalent among household cats. FHV-1 is frequently lethal for kittens, while adult cats typically have the ability to survive and maintain a persistent infection throughout their lives. The primary clinical manifestations of FHV-1 infection in feline hosts include conjunctivitis, keratitis, and upper respiratory illness, with pneumonia as an infrequent consequence (14). It is recognized as the predominant culprit accountable for feline ocular surface illness. Corneal ulceration in a cat is typically a secondary indication of the presence of FHV-1 infection (15). Feline Calicivirus (FCV) is a highly prevalent infection that is widely distributed across the feline population. It can even be found in carriers who show no symptoms. This infectious virus can result in a range of clinical complications (16).

Indeed, diagnosing FHV-1 can be a challenge in adult cats. A repetitive history of eye inflammation that keeps on coming back or corneal ulcers possibly with sneezing may indicate infection. In the course of initial stages, huge numbers of neutrophils show up when one examines eye discharge under microscope. These cells contain lymphocytes, plasma cells and a few eosinophils sometimes as well as mast cells in some instances that are seen with chronic infections. Infections caused by viruses are characterized by numerous neutrophils but no viral particles are visible within the cells (17). Some definitive tests for FHV-1 and FCV include virus isolation, fluorescent antibody staining, and PCR. Out of these methods, PCR is currently considered the most commonly used method for studying samples from eyes and throats on both animals and humans alike. While being highly sensitive and specific, variations may occur in results obtained using PCR depending on laboratories used (17).

Former studies on ocular microorganisms in animals have been based on traditional culture approaches including swabs taken from cornea and conjunctiva followed by biochemical testing and mass spectrometry to identify bacteria grown. Those common Gram-positive bacteria found in normal cat eyes consist of *Staphylococcus, Streptococcus,* and *Corynebacterium* among others (13). *Staphylococcus epidermidis* is a Gram-positive bacterium that belongs to the group of coagulase-negative bacteria. It is considered an opportunistic pathogen. Coagulase negative staphylococci (CoNS) naturally reside on the skin, mucosal, and ocular surfaces of humans, and are responsible for infections acquired in hospitals. This organism has the potential to induce ocular ill-

nesses such as bacterial endophthalmitis, conjunctivitis, blepharitis, and keratitis (18).

The pathogenic fungal species and the visual result of eye infection exhibit diversity. Fungal eye infections are common in horses but uncommon in ruminants, dogs, and cats (10). The widespread occurrence of fungi on the conjunctiva and cornea of other animals, together with possible causation of corneal infection. The predominant fungal organisms found in healthy eyes are *Cladosporium*, *Alternaria*, *Fusarium*, *Aspergillus* spp., and *Penicillium* spp. In contrast, the most commonly identified fungal isolates in diseased animal eyes are *Aspergillus* spp., *Fusarium* spp., and *Candida* spp., with variations depending on the specific location (18).

In the current study, an attempt has been made to study the microbiota and the frequency of different types of microorganisms in the eyes of these groups by examining the flora of the eyes in cats infected with Herpesvirus and Calcivirus, as well as Herpes/ Calcivirus.

MATERIALS AND METHODS

Animals. The study was performed on 240 selected cats that had been visited in a veterinary health centers (Tehran, Iran) for examination between 2021 and 2022. All cats underwent a comprehensive eye examination conducted by a veterinary ophthalmologist to assess their ocular health. This stage included the assessment of the anterior part of the eye by slit lamp biomicroscopy and the posterior part of the eye by indirect ophthalmoscopy. A routine minimal ophthalmic test that included fluorescein staining and tonometry was carried out.

Microbiota assessment. Sterile swabs from each cat's eyes were inoculated into nutrient broth and immediately transported to the research institution, where they were incubated at 37°C for 24 hours and investigated for microbiological assessment. Samples were cultured on Blood Agar, Nutrient Agar, and MacConkey Agar and then incubated for 48 hours at 37°C. Bacterial isolates were examined using Gram's stain method and biochemical (oxidase, catalase, coagulase, TSI, and carbohydrates fermentation) tests (19). Growth media was used for fungal samples: Sabouraud Dextrose Agar (for 96 hours at 25°C) and fungus investigated microscopically.

Viral pathogens identification by PCR. PCR assay was performed on cats exhibiting clinical indications of conjunctivitis. Prior to fluorescein staining, samples of eye flora were obtained in cats to avoid any contamination or dilution of the material. The process of collecting samples in cats involved applying a single drop of 0.5% proparacaine to the surface of each eye to offer local pain relief. Specimens were obtained from the inferior conjunctival fornix of each cat's eyes at three distinct time intervals. A total of two isohelix buccal swabs were employed for each eye, with each side of the swab being gently massaged in the inferior conjunctival fornix ten times.

A total of 240 conjunctival swabs were evaluated for the detection of pathogens by PCR and subsequently, cats were classified into 4 groups based on their health state (normal, infected with different pathogens herpes, clasi, and herpes/calsi). Each group was accompanied by data relating to cat sex and age.

DNA extraction. DNA extraction for FHV-1 PCR was performed using the QIAamp DNA Mini Kit from Qiagen, located in Hilden, Germany. DNA extraction was performed by taking 200 microliters of either a swab sample or cell culture supernatant. The manufacturer's protocol was followed, using spin and vacuum procedures appropriate for eye, nasal, or pharyngeal swab samples. Viral RNA was extracted using the QIAamp RNA Mini Kit from Qiagen. 140 microliters of either swab sample or cell culture supernatant were taken. The manufacturer's protocol was followed, utilizing spin and vacuum procedures to purify viral RNA from eye, nasal, or pharyngeal swab samples (20).

PCR amplification of FHV-1 and FCV. The primers and PCR conditions used for FHV-1 detection were adapted from the protocol originally developed by Sykes et al. (21). The FHV-1 PCR amplification was performed in a 50-microliter reaction volume. The reaction contained 4mM $MgCl_2$, 200μM each dNTP blend (from Applied Biosystems), 0.4μM of forward (5'-GACGAGTTTCCCGTCTACCG-3') and reverse (5'-GACGCTTAGGCGGTGTTGGG-3') primers each, 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), the appropriate amount of PCR buffer, RNase-free water, and 5μL of extracted sample template. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at

94°C for 60 seconds, primer annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step of 7 minutes at 72°C was performed. This PCR yields a 287 bp amplicon (20).

The primers and PCR conditions used for FCV detection were adapted from the protocol originally developed by Sykes et al. (21). For the FCV RT-PCR, 5 microliters of extracted sample were first added to 45 microliters of MasterMix in the PCR tube, using the Roche Titan One Tube RT-PCR System (Sigma). The 50-microliter reaction contained 0.4 micromolar each of the forward (5'-GCCAGAAGATGGTGTTGAGG-3') and reverse (5'-GCTTCTGCTTCTGTTGTCAC-3') primers, 200 micromolar of each dNTP blend, 2.5 units of enzyme mix, 2.5mM magnesium chloride, the appropriate amount of PCR buffer, RNase-free water, and the 5 microliters of template. After an initial reverse transcription step at 42°C for 60 minutes, PCR amplification was performed for 40 cycles of 95°C for 60 seconds, 56°C for 60 seconds, and 72°C for 30 seconds, followed by a final extension of 7 minutes at 72°C. This yielded an amplicon of 670 to 680 base pairs (20).

Analysis of PCR products. The PCR products were examined by electrophoresis on a 1.5% agarose gel in 1× TBE buffer. The gel was electrophoresed at a voltage of 80V (for 8 channels) or 100V (for 18 channels) for a duration of 50 minutes. Following electrophoresis, the gel was treated with SYBR Safe (Invitrogen) in TBE buffer to enhance the visibility of the bands. The molecular weight standard utilized was a 1kb Plus DNA ladder from Invitrogen, with a size of 20.

RESULTS

Demographical data. The results of the evaluation of demographic characteristics are reported in Table 1. As shown in this Table, there was a significant difference between the age of the studied samples in the normal and herpes groups compared to the Calsi group (P=0.006).

Microbiota assessment. The most common bacterial isolates are *S. intermedius* and *S. agalactiae* and the lowest frequency was related to the three species *E. coli* non-hemolytic, *B. flexus*, and *S. haemolyticus*. The diagram of bacterial frequency in the normal group is shown in Fig. 1. In addition, the evaluation of fungal frequency in the normal group indicated the prevalence of 3 cases of *Aspergillus fumigatus* and 3 cases of yeast. The prevalence of bacteria was substantially greater than the prevalence of fungi (P<0.0001).

The investigation of the frequency of bacteria in the Herpes-infected group showed that the highest frequency was related to the species *S. epidermidis*, and the lowest frequency was observed for the species *Corynebacterium xerosis*. The graph related to bacterial Frequency in the Herpes-infected group is shown in Fig. 2. In addition, the evaluation of the frequency of fungi in this group showed that the highest and lowest prevalence belonged to Yeast and *Microsporum persicolor*, respectively (Fig. 3). Statistical comparison between bacterial and fungal prevalence in this group showed that bacterial frequency was significantly higher than fungal organisms (P<0.0001).

The evaluation of bacterial frequency in the Calsi-infected group indicated that the most common species is *S. epidermidis* (with the highest frequency) and, in contrast, *C. xerosis* species obtained the lowest frequency value (Fig. 4). Moreover, as shown in Fig. 5, Yeast species and *Microsporum persicolor/Cladosporium* species in the Calsi-infected group showed that the highest frequency was related to and had the highest and lowest frequency, respectively. Statistical analysis in this group showed that bacterial frequency was significantly more than fungal frequency (P<0.001).

The examination of bacterial frequency in the Herpes/Calici-infected group showed that the *S. intermedius* species has the most common frequency and the lowest frequency belonged to *Pseudomonas aeruginosa* and *Enterobacter* species (Fig. 6). In addition,

Table 1. Frequency distribution of demographic characteristics of the samples

Variable		Normal	Herpes	Calsi	Herpes/calsi
Age/months		0.77 ± 4.15	0.77 ± 4.15	0.64 ± 3.71	0.68 ± 3.93
Sex	Female	33 (55%)	23 (38.33%)	33 (55%)	27 (45%)
	Male	27 (45%)	37 (61.67%)	27 (45%)	33 (55%)

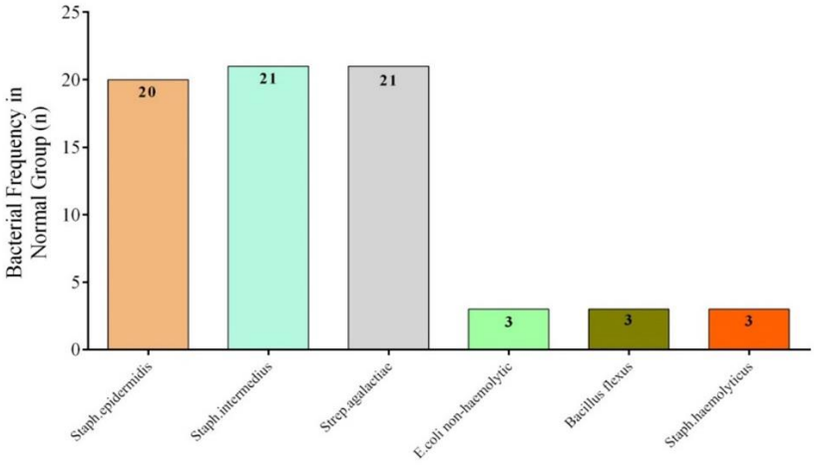

Fig. 1. Frequency of bacterial species in the normal group

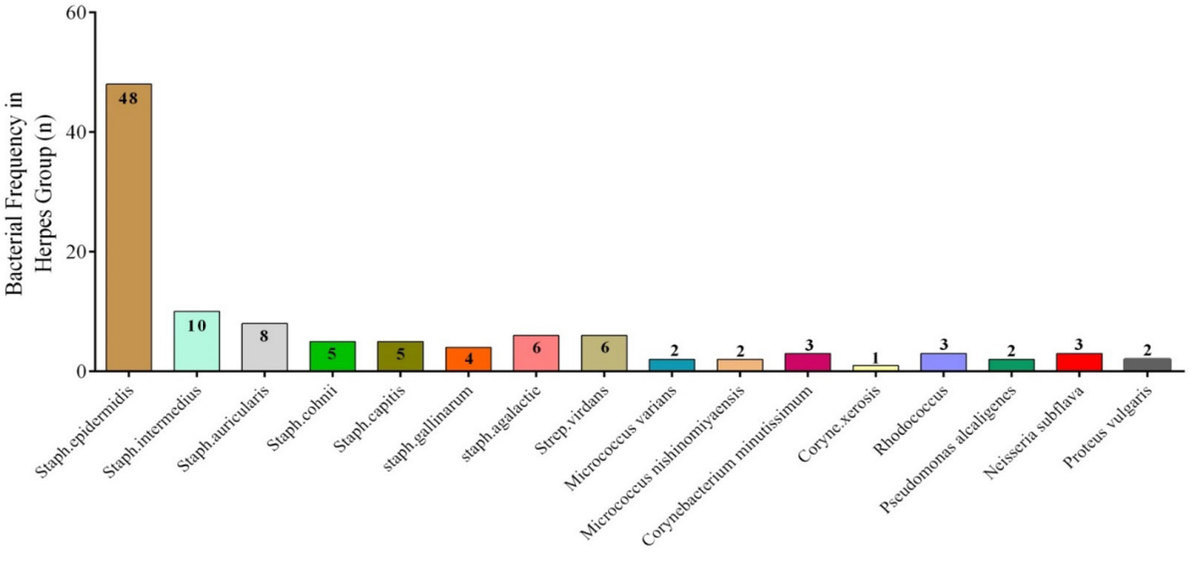

Fig. 2. Frequency of bacterial species in the Herpes-infected group.

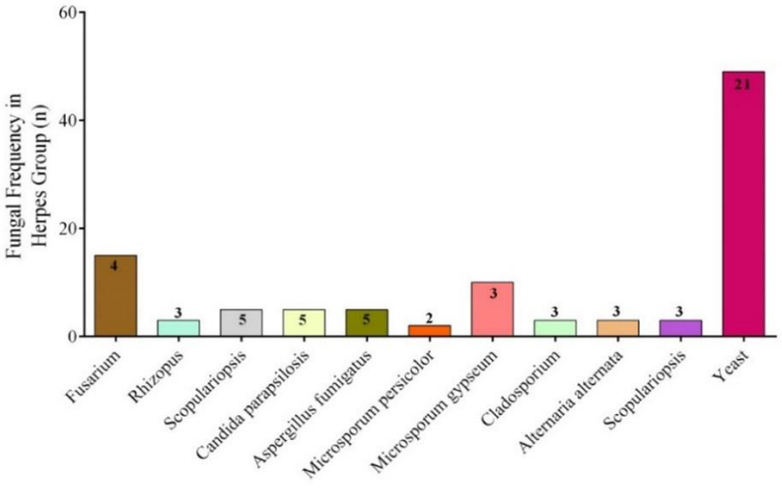

Fig. 3. Frequency of fungal species in the Herpes-infected group.

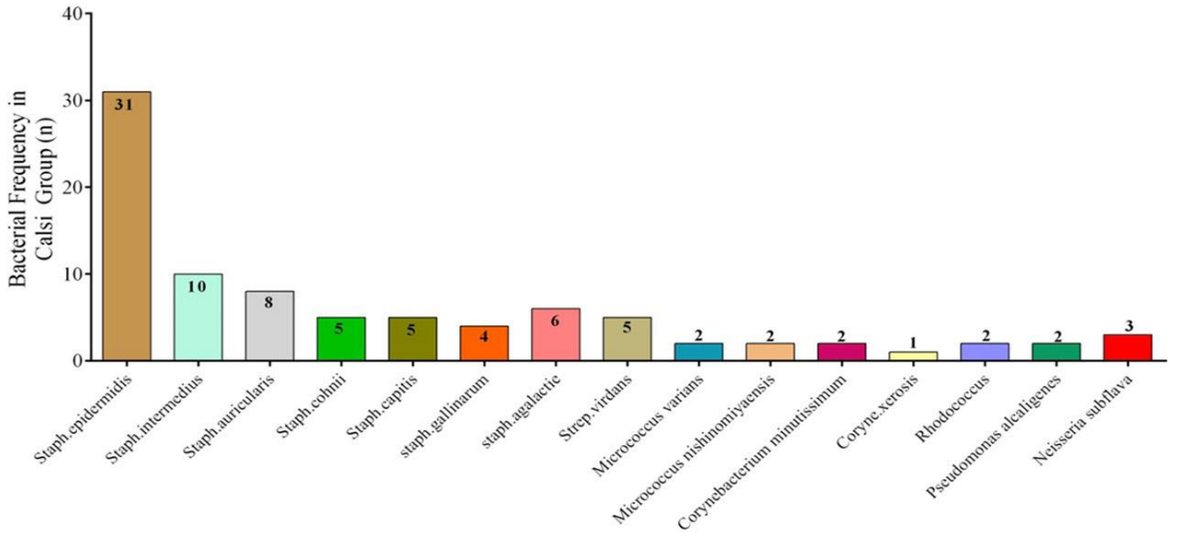

Fig. 4. Frequency of bacterial species in Calsi- infected group.

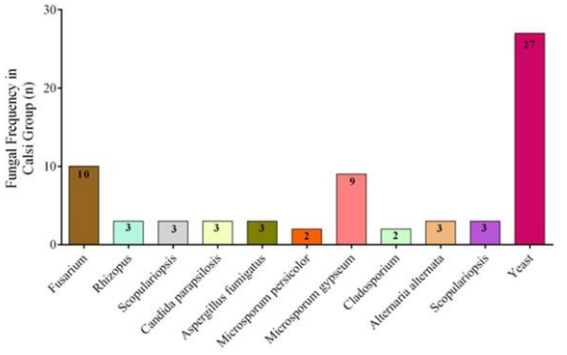

Fig. 5. Frequency of fungal species in the Calsi-infected group.

the assessment of fungal prevalence in this group indicated that the highest frequency was related to *Aspergillus fumigatus*, *Aspergillus niger*, and *Penicillium* species, while *Candida parapsilosis*, *Microsporum gypseum*, and *Chaetomium* species obtained the lowest frequency value (Fig. 7). As shown in Fig. 8, The prevalence of bacteria was extensively greater than the prevalence of fungi in this group ($P<0.0001$).

DISCUSSION

The ocular surface microbiota consists of non-pathogenic microorganisms that inhabit the conjunctiva and cornea. The microbiota of the ocular surface appears to be mostly composed of Gram-positive and Gram-negative bacteria, as well as several kinds of fungi (9). The ocular surface is continuously exposed to the external environment and many conditions, making it susceptible to infection with pathogens. Throughout the course of evolution, several types of microorganisms, such as bacteria and fungi, have established themselves as commensals on the surface of the eye, creating microbiota (9). Due to the high prevalence, importance, and definite effect of this disease on the microbial flora of the eyes of affected cats, before this study, there has not been a study in the form of a specific investigation of bacterial and fungal changes in cases of these diseases.

Aftab et al. (2019) showed during the study that most bacteria isolated from the eyes of cats were Gram-positive bacteria. The most commonly isolated bacterial organisms were *Staphylococcus epidermidis* (41/95; 43.2%), β-hemolytic streptococci (18/95; 18.9%), *Staphylococcus aureus* (17/95; 17.9%), and *Escherichia coli* (11/95; 11.5%) (22). While in the present study, in the normal group, two bacterial species, *S. intermedius* and *S. agalactiae*, in the Herpes- and Calici-infected groups, *S. epidermidis*, and in the last group (Herpes/Calici-infected), *S. intermedius* showed the highest frequency. In addition, Buttner et al. (2019) isolated microorganisms from 49 of 120 cats (40.8%) and 73 of 240 swabs (30.4%). Of the isolates, 71% (61/86) were Gram-positive bacteria, 26% (22/86) were Gram-negative bacteria, and 3% (3/86) were fungi. *S. felis* (17/86; 19.8%) was the most commonly isolated species and *Moraxella oslo-ensis* (5/86; 5.8%) was the most frequent Gram-negative species (23).

Furthermore, prior investigations have identified

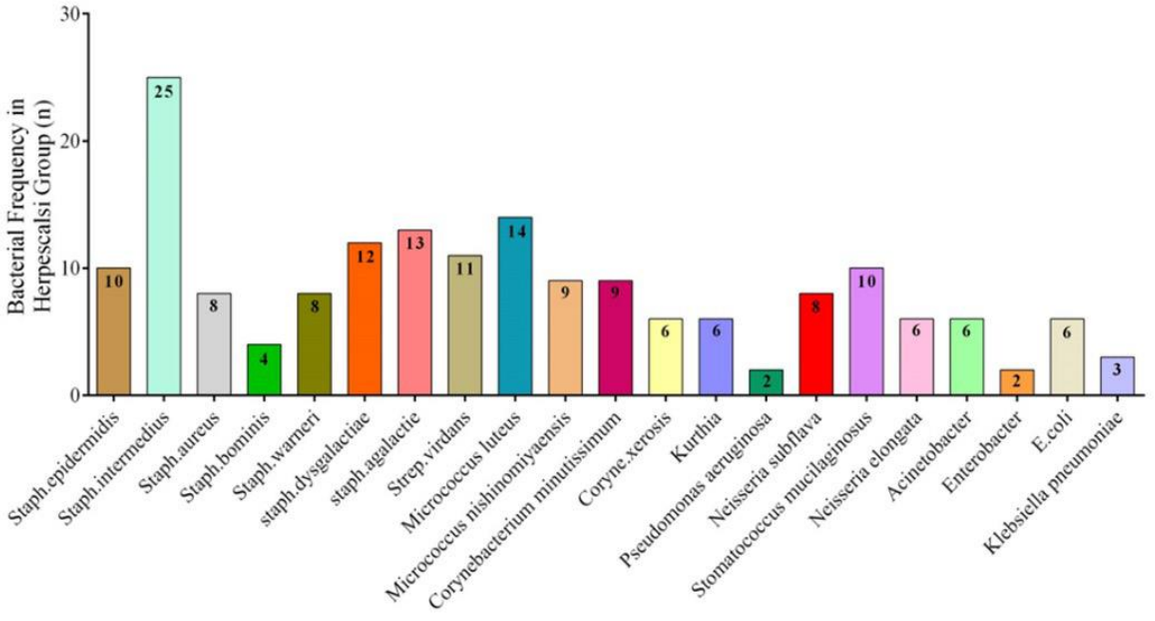

Fig. 6. Frequency of bacterial species in Herpes/Calici group.

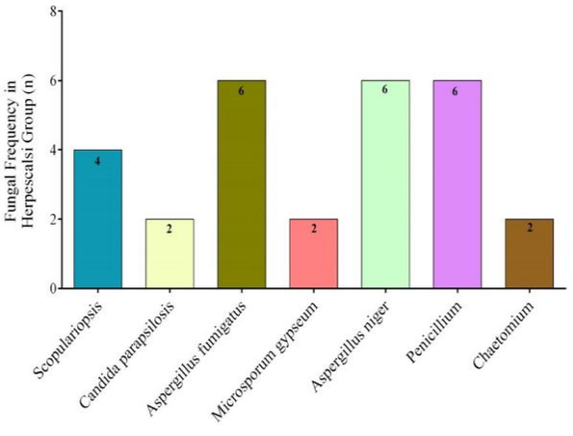

Fig. 7. Frequency of fungal species in Herpescalsi group

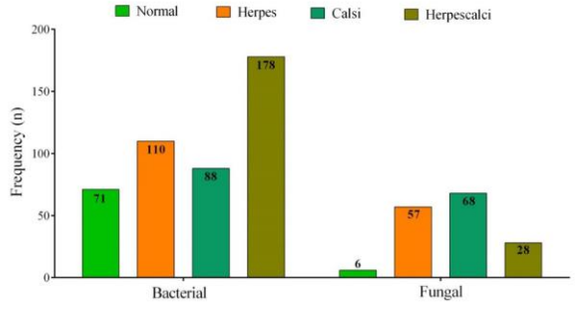

Fig. 8. Overall Frequency of bacterial and fungal species in the Herpes/Calici-infected group.

several kinds of fungi that were found in the normal conjunctiva of animals. These include *Mucor* spp., *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., and *Cladosporium* spp. (11). In The present study, the fungi that demonstrated a high frequency in the samples (normal and viral-infected cats) included different species of *Aspergillus (Aspergillus fumigatus, Aspergillus niger), Penicillium* and yeast. In another study, the fungal species was identified and these species contain *Aspergillus flavus, A. fumigatus, A. niger, Penicillium* spp., *Mucor* spp., and *Alternari* spp. with a corresponding prevalence rate of 63.9%, 27.8%, 15.3%, 18.1%, 13.9%, and 4.2%, respectively, in healthy animal eyes, while their prevalence in diseased animal eyes was 57.1%, 32.1%, 21.4%, 7.1%, 3.6%, and 0% (18). In addition, during the study conducted by Samuelson et al., fungi were also isolated from healthy eyes of cats (40%). The conjunctiva of these animal species was colonized by a subset of fungal species including *Aspergillus* spp. in 8% of the cats. *Penicillium* spp. and *Cladosporium* spp. were isolated from these animals (24).

CONCLUSION

The statistical analysis comparing the study groups in terms of microbial and fungal frequency revealed a

substantial disparity. Specifically, the frequency was found to be higher in all disease groups compared to the normal group ($P<0.05$). In the comparison between the disease groups, the results indicated that the Herpes/Calici group was significantly higher and lower in bacterial and fungal prevalence compared to the Herpes-infected and Calici-infected groups (both groups $P<0.0001$). Moreover, a significant relation was observed between the Herpes-infected and Calici-infected groups regarding microbial and fungal prevalence, such that the group infected with Herpes exhibited a higher prevalence of microorganisms and a lower prevalence of fungi compared to the Calici group ($P<0.001$ and $P<0.01$, respectively).

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