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Original article

PREVALENCE OF *MALASSEZIA* SPP. IN THE EXTERNAL EAR CANALS OF DOGS FROM GORGAN, IRAN AND ANALYSIS OF SOME PREDISPOSING FACTORS

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Summary

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Yeasts of the genus *Malassezia* grow in areas with sebaceous glands like ears due to their reliance on lipids. This study aimed to identify the prevalence and population size of different species of *Malassezia* in the external ear canal of dogs. Additionally, possible correlations between the occurrence of *Malassezia* yeasts and the sex, age, breed, and skin/ear disease history of the dogs were assessed. One hundred ear swabs from both ears of 50 dogs were collected. The collected samples were directly observed by the KOH wet mount method under the microscope. Then, they were subjected to fungal culture and incubated. Staining was done by the Gram method. The prepared spreads were observed and examined with an x100 microscope lens. For identifying yeast colonies, three biochemical tests containing catalase, different percentages of Tween, and aesculin hydrolysis were used. PCR molecular test was used to confirm the diagnosis of the identified yeast species and its data was matched with the identified cases. The prevalence of *M. pachydermatis* and *M. furfur* was 44% and 6% respectively. Infection by *Malassezia* species was most prevalent in the native breed (78.26%). There was a higher incidence of *Malassezia* yeast contamination in female dogs (60.71%). The frequency of fungal species in dogs more than 2 years old was higher. There was a significant difference between the prevalence of *Malassezia* species frequency and the breed of the dogs (P=0.003) but no significant differences were found correlated with sex, age, and skin/ear disease history of the dogs.

Key words: dogs, Iran, *Malassezia pachydermatis*

INTRODUCTION

The genus *Malassezia* is made up of yeasts with a thick cellular membrane and multiple layers (Nobre *et al.,* 2001). *Malassezia* yeasts do not form mycelium and reproduce asexually through unipolar budding (Cafarchia *et al.,* 2005). There are 18 known species including *M. furfur, M. pachydermatis, M. sympodialis, M. globosa, M. obtusa, M. slooffiae, M. restricta, M. dermatis, M. japonica, M.*

nana, M. yamatoensis, M. caprae, M. equina, M. cuniculi, M. arunalokei, M. brasiliensis, M. psittaci, M. vespertilionis. Most *Malassezia* species are unable to synthesise C14 or C16 fatty acids (Guillot & Bond, 2020). Therefore, they grow in areas with sebaceous glands due to their reliance on lipids (Kindo *et al.,* 2004). Different species have varying levels of lipid dependence, which has led to the utilisation of specific tests for their identification (Guillot & Bond, 2020). *Malassezia* yeasts are a common cause of canine otitis externa, with a prevalence as the sole causative agent ranging from 8% to 26% (Tešin *et al.,* 2023).

M. pachydermatis is the only species in the genus that does not depend on lipids for growth. It appears as isolated or grouped cells with an oval shape or a "bottle" shape due to budding. Hyphae and pseudohyphae are typically not present (Nobre *et al.,* 2001). *M. pachydermatis* has different genetic subtypes that may be specific to certain hosts. It is important to note that *Malassezia* is a diverse group of species with different genotypes that can cause similar skin conditions (Theelen *et al.,* 2018). *M. pachydermatis* is commonly found on the skin of dogs and is considered a normal part of their microflora. However, it can also become opportunistic and cause skin issues in dogs with seborrhoeic dermatitis and ceruminous otitis externa (Nardoni *et al.,* 2004). It's frequently found in 30% to 80% of otitis externa cases and 30% of seborrheic and atopic dermatitis cases (Kumar *et al.,* 2002).

Otitis externa is a common disease in dogs, with a prevalence rate ranging from 10% to 20% (Campbell *et al.,* 2010). The canine auditory canal is susceptible to changes that can lead to the growth of microorganisms (Fernández *et al.,* 2006). *M. pachydermatis* is found in 15% to 50% of healthy ears and can increase to 83% in infected ears (Lyskova *et al.,* 2007). Other types of *Malassezia* yeast can also contribute to otitis, with more pathogenic strains being found in dogs with the condition (Bardshiri *et al.,* 2014). In dogs, the shape of the ear canal is a predisposing factor associated with otitis externa. Most authors agree that long-haired, pendulous-eared breeds are more susceptible to otitis externa compared to dogs with erect ears (Cafarchia *et al.,* 2005; Lehner *et al.,* 2010; Kaimio *et al.,* 2017).

The purpose of this study was to identify the prevalence and population size of different species of *Malassezia* in the external ear canal of dogs. Additionally, possible correlations between the occurrence of Malassezia yeasts and the sex, age, breed, and skin/ear disease history of the dogs were assessed.

MATERIALS AND METHODS

Sampling procedure

This study was conducted from September 2022 to September 2023 in Gorgan, Iran, and samples were gathered from different private veterinary clinics and dog shelters around the city. During this period, 100 samples from both ears of 50 dogs were collected. They included 22 male dogs aged 1.09±1.0 years (mean±SD) and 28 female dogs aged 1.4±2.1 years (mean± SD) were studied. Twenty-four dogs were less than 2 years old and the other 26 dogs were more than 2 years old.

The following dog breeds were sampled in this study: Native (23), Shih tzu (6), Maltese (4), Spitz (2), Golden retriever (2), Poodle (2), Husky (1), Pitbull (1), Corgi (1), Samoyed (1), Cocker spaniel (1), Pomeranian (1), Labrador retriever (1), Bulldog (1), German shepherd (1), Spanish pointer (1), and Chihuahua (1).

Samples were taken by sterile cotton-tipped swabs, moistened in distilled water, and rubbed in the external ear canal. In addition, data of the sex, age, breed, and skin/ear disease history of the examined dogs was taken from their owners.

Laboratory methods

The collected samples were directly observed by the KOH wet mount method under the microscope. The swab specimens were separately inoculated onto Sabourad's dextrose agar (Condalab, Madrid, Spain), modified Dixon's agar (Ibresco, Karaj, Iran), and Sabouraud's dextrose agar with chloramphenicol and cycloheximide (Condalab, Madrid, Spain) plates. The plates were incubated at 37°C for 14 days. After the growth of fungal colonies, checking and registering the morphological characteristics of the fungal colonies, staining was done by the Gram method. In the next step, the prepared spreads were observed and examined with an x100 microscope lens. Impure samples were purified and pure fungal colonies were obtained.

Biochemical tests

In order to identify yeast colonies, three biochemical tests containing catalase, different percentages of Tween, and aesculin hydrolysis were used and their results were interpreted based on the relevant diagnostic properties and characteristics of Malassezia species table represented by Ashbee (Ashbee, 2007). The catalase reaction was determined by the application of a drop of hydrogen peroxide (10 vol.) onto a portion of a colony on a glass slide. The production of gas bubbles indicated a positive reaction. Different percentages of Tween including Tween 20 (10%), Tween 40 (0.5%), and Tween 80 (0.1%) were added to the Dixon's agar plates, and yeast fungal samples were inoculated onto these plates. Utilization of Tweens was assessed by the degree of growth and/or reaction (precipitation) of the lipophilic yeasts around individual wells. The ability of isolates to split aesculin to produce aesculetin and glucose was tested. In this test, aesculin is incorporated into an agar medium, and the production of aesculetin is indicated by its reaction with iron salt to give a dark-brown, black color in the medium. The blackening of the colony and the surrounding medium denoted a positive reaction.

PCR

PCR molecular test was used to confirm the diagnosis of the identified yeast species. Yeast genomic DNA isolation was performed based on the Bust n' Grab protocol (Harju *et al.,* 2004). The PCR amplification was performed in a total volume of 25 µL reaction mixtures containing 100 ng DNA template, 10 µL of Taq DNA Polymerase Master Mix RED (Ampliqon, Odense, Denmark), and 0.5 µL of each primer (10 pM). For identifying the genus of the yeasts, ITSANA-F (5′-CGAAAC GCGATAGGTAATGTG-3′) and ITSA NA-R (5′-CAAATGACGTATCATGCCA TGC-3′) with ampilicon size of 340 bp were used as primer pair. Multiplex PCR method was used to identify the species. In this method, ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS3 (5′-GCAT CGATGAAGAACGCAGC-3′) were forward primers, and ITS2 (5′-GCT GCGTTCTTCATCGATGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′): reverse primers. Also, in order to identify the species of the product obtained from each pair of primers, the primers were blasted in different pairing conditions using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiplex

pattern of the obtained amplicons was compared with the BLAST results and the species of each sample were identified. The PCR thermal conditions for genus identification were 95 °C for 2 min; followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 45 s; and a final extension at 72 °C for 10 min. The PCR thermal conditions for species identification were 95 °C for 2 min; followed by 40 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 1 min; and the final extension at 72 °C for 10 min. The PCR products were visualised by 1.5% (w/v) agarose gel electrophoresis in TBE buffer, stained with SYBR Safe DNA gel stain (1:10,000 dilution in TBE), and photographed under ultraviolet transilluminator (UVITEC, UK). The expected fragment sizes for *M. furfur* were 827 bp, 557 bp, and 284 bp, whereas the expected fragment sizes for *M. pachydermatis* were 776 bp, 530 bp, and 266 bp.

Statistical analysis

The following data were analysed: the number of *Malassezia* species in dogs' ears and the relationship between sex, breed, age, skin/ear disease, and *Malassezia* species in the ears of dogs. Data were analysed by the chi-square test. A P value of <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS software version 24 (IBM Corp, Armonk, NY).

RESULTS

Malassezia species in the studied population. *M. pachydermatis* was isolated from 25 dogs (50% of ear samples) and *M. furfur* isolates were found in 3 dogs (6% of ear samples).

The *Malassezia* species based on culture-based and biochemical-based methods were *M. pachydermatis* and *M. furfur* (Fig. 1 and 2). The results from the PCR test also confirmed the existence of *M. pachydermatis* and *M. furfur* species (Fig. 3).

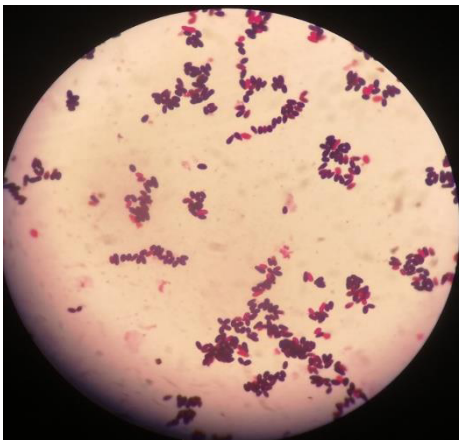

Fig. 1. *M. pachydermatis* with cylindrical cells, broad base, and distinct bud scar (Gram stain, ×100).

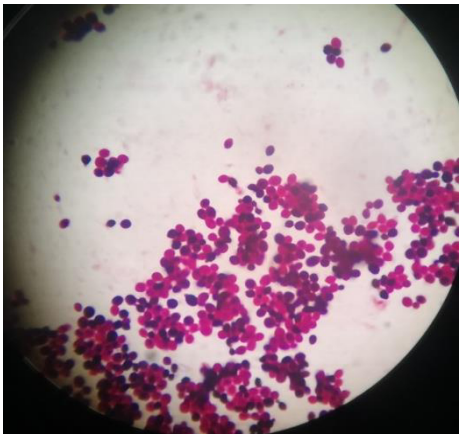

Fig. 2. *M. furfur* with small ovoid to spherical cells and a broad base (Gram stain, ×100).

The statistical analysis figured out 11 (50%) positive *Malassezia* cases in male dogs and 17 (60.71%) positive cases in female dogs. For the variety of breeds, they were divided into two groups including native breed and other breeds. The

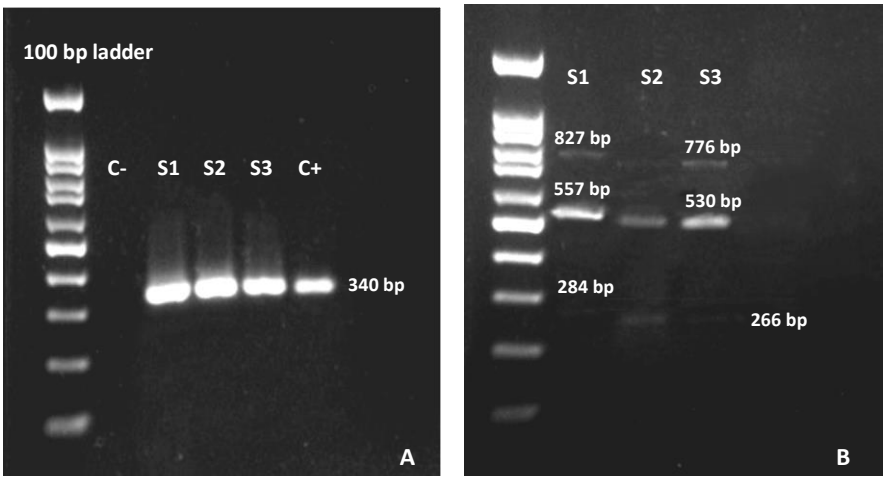

Fig. 3. Agarose-gel electrophoresis for evaluation of PCR products. **A.** Lane C-: negative control, lane C+: positive control, lanes S1, S2, S3: sample 1, 2, 3. The expected 340 bp fragment size for *Malassezia* genus is visualised. **B.** Lane S1: the expected fragment sizes for *M. furfur* were 827 bp, 557 bp, and 284 bp (ITS1-ITS4: 820–827 bp, ITS3-ITS4: 469–577 bp, ITS1-ITS2: 284–290 bp); lane S3: the expected fragment sizes for *M. pachydermatis* were 776 bp, 530 bp, and 266 bp (ITS1-ITS4: 776 bp, ITS3-ITS4: 512–530 bp, ITS1-ITS2: 266–273 bp).

results showed a *Malassezia* prevalence of 78.26% in the native breed and 37.04% in other breeds. In addition, the prevalence of *Malassezia* species in dogs aged more than 2 years was higher. *Malassezia* contamination was detected in 8 (72.73%) dogs with skin/ear disease history which was significantly higher than in dogs without a history of skin/ear disease. There were no statistical differences for sex (P=0.459), age (P=0.422), and skin/ ear disease history (P=0.214) in relation to the isolation of *Malassezia* species, but there was a significant difference between the prevalence of *Malassezia* and dog breed (P=0.003). The distribution of *Malassezia* species frequency based on recorded factors is represented on Fig. 4.

DISCUSSION

M. pachydermatis is known as the most important fungal species involved in otitis externa. Also, in previous studies it is known as the most prevalent isolated yeast species (Yoshida *et al.,* 2002; Fernández *et al.,* 2006; Girão *et al.,* 2006; Lyskova *et al.,* 2007; Deneva *et al.,* 2020). The results of the present study are in agreement with the previous researches.

Malassezia species, particularly *M. pachydermatis*, can be found in dogs of all breeds, with some breeds being more commonly affected. Nardoni *et al*. (2004) reported the highest prevalence of *Malassezia* in crossbred dog, whereas Poodle and German Shepherd breeds were the dominant breeds found to harbour *Malassezia* in another study (Girão *et al.,* 2006). *M. pachydermatis* was more prevalent in Terriers and Iranian Mix Shepherds breeds (Bardshiri *et al.,* 2014). In the present research, *Malassezia* species had the greatest prevalence in the native breed (78.26%).

The distribution frequency of *Malassezia* species has been reported by many

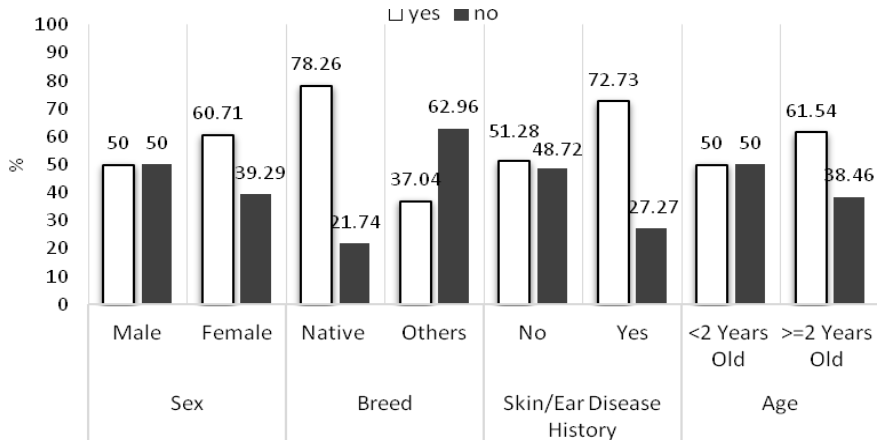

Fig. 4. Distribution of *Malassezia* species frequency based on studied factors.

researchers. It is evident that *M. pachydermatis* is the most commonly found species in canine ears in agreement with the results of the current study (Keshin *et al.,* 2010; Karlapudi, 2017; Deneva *et al.,* 2020; Núñez *et al.,* 2022; Al-Shuwaili *et al.,* 2023). *M. furfur* strains were detected in the present study, in line with reported *M. furfur* by Crespo *et al.* (2002), Nardoni *et al.* (2004), Deneva *et al.* (2020). Other species of *Malassezia* genus have been reported in a few previous studies. Although *M. sympodialis* was not found in our research observations, Kindo *et al.* (2004) reported this species as the second most prevalent *Malassezia* yeast in dogs' ears.

The frequency of *M. pachydermatis* has been analysed in different research studies based on different age ranges of dogs. Positive *M. pachydermatis* results were more common in dogs from 1 to 8 years of age (Bardshiri *et al.,* 2014), whereas Girão *et al*. (2006) reported higher frequency of this species in dogs of from 1 to 3 years of age. In our study, *M. pachydermatis* was more prevalent in dogs > 2 years of age.

In many studies, no significant difference is found between *Malassezia* species prevalence and dogs' sex, age, and breed (Nobre *et al.,* 2001; Bardshiri *et al.,* 2014; Núñez *et al.,* 2022) but we found a significant difference between *Malassezia* species prevalence and breed of the dogs. Moreover a very significant difference ($P<0.01$) in *Malassezia* species frequency was reported in dogs having ear disease history (Nardoni *et al.,* 2004; Lyskova *et al.,* 2007; Bardshiri *et al.,* 2014) in contrast to our study results.

A higher prevalence of *M. pachydermatis* was reported in dogs with pendulous ears (Bardshiri *et al.,* 2014) in contrast with results from abother research (Campbell *et al.,* 2010).

CONCLUSION

In conclusion of the conducted research, *M. pachydermatis* was determined to be the most prominent fungal species in dog ear contamination. Statistical analysis showed a significant difference in the prevalence of *Malassezia* species relating to dog breed, but no significant differ-

ences were found in relation to sex, age, and skin/ear disease history of the dogs. These results can be useful in the detection and treatment of dog ear fungal diseases.

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