OCCURRENCE OF *MALASSEZIA* YEASTS IN DERMATOLOGICALLY DISEASED DOGS

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ABSTRACT

The *Malassezia* genus is represented by several lipophilic yeasts, normally present on the skin of many warm-blooded vertebrates, including humans. The aim of this study was to investigate the occurrence of *Malassezia* yeasts in dogs with skin lesions (dermatitis, interdigital dermatitis and inflammation of anal sacs) and otitis externa. The presence of *Malassezia* spp. was investigated in a group of 300 dogs exhibiting clinical manifestations. The isolates of *Malassezia* were identified by using phenotypic (biochemical-physiological and morphological characteristics) and genotypic methods (PCR, RFLP-AluI, BanI and MspA1I) which allowed their precise identification. *Malassezia* yeasts were isolated from 84 specimens obtained from 76 positive dogs. *M. pachydermatis* was the most frequently isolated species (79 isolates) in this study. *M. furfur* was identified in four dogs and *M. nana* in one dog. The prevalence of isolated *Malassezia* spp. was 25.3 % in dogs with skin lesions; from which 36.0 % were dogs suffering from otitis externa, 24.5 % from dogs having dermatitis, 16.4 % from dogs with interdigital dermatitis and 14.3 % from dogs having inflammation of the anal sacs. A higher prevalence of *Malassezia* spp. was observed in animals with pendulous ears in comparison with dogs having erect ears.

Key words: dermatitis; diagnostics; dogs; *Malassezia*; otitis externa

INTRODUCTION

Canine otitis externa and dermatitis are frequently encountered diseases in a veterinary practice. The etiology of canine otitis externa is complex and involves many factors which can be classified as: predisposing, primary and perpetuating. Microorganisms, both bacteria and yeasts, are considered perpetuating factors [2]. The associated microflora of otitic ears include most often bacteria (Staphylococcus spp., Pseudomonas aeruginosa, *Proteus* spp., *Steptococcus* spp.) and yeasts (*Malassezia* spp., *Candida* spp. and other) [18]. *Malassezia* yeasts also play an important

role in the development of seborrheic, atopic and allergic dermatitis in dogs. Yeasts are known to be more frequently found in areas where there is an ample amount of sebum secretions [6]. These lipophilic yeasts may be isolated from normal ear canals and also healthy skin, but if environmental conditions are suitable, the otitis externa or dermatitis can be created by these pathogens [9]. So far, 16 species of *Malassezia* are known: *M. dermatis*, *M. japonica*, *M. obtusa*, *M. restricta*, *M. yamatoensis*, *M. furfur*, *M. globosa*, *M. slooffiae*, *M. sympodialis*, *M. pachydermatis*, *M. caprae*, *M. equina*, *M. cuniculi*, *M. nana*, *M. brasiliensis* sp. nov and *M. psittaci* sp. nov. [3]. Up to now, only *M. pachydermatis* [1, 4], *M. furfur* [10, 11, 13] and *M. obtusa* in participation with *M. furfur* [10] have been identified in dogs. The preliminary diagnosis of *Malassezia* dermatitis and otitis is suggested by the typical clinical findings, such as erythema, greasiness, alopecia, lichenification, variable hyperpigmentation and marked pruritus, and by the identification of yeasts in specimens obtained from affected areas, which demonstrate the lack of response to treatment with antibiotics, corticosteroids and immunotherapy [20]. The definitive diagnosis is based upon yeast identification composed of phenotypic and genotypic methods. The phenotypic methods (biochemical and morphological characteristics) are time-consuming and are subject to variable interpretation. Genotypic identification (fingerprinting methods, DNA sequence analysis and restriction analysis of PCR amplicons) is necessary for the exact diagnosis [29].

The aim of this study was to identify *Malassezia* yeasts in dermatologically diseased dogs and to determine their prevalence.

MATERIALS AND METHODS

The survey was carried out on 300 dogs with skin lesions, otitis externa or inflammation of the anal sacs (174 male and 126 female). The ages of the animals ranged from 8 weeks to 14 years. The samples were collected from affected body sites (external ear canals, interdigital areas, cutaneous lesions, and anal sacs) of dogs with clinical manifestation, by using sterile cotton swabs (Fungi-Quick, Dispolab, SR). The samplings were acquired before employing any antimicrobial therapy, particularly from the affected areas with symptoms, such as seborrhea, erythema, alopecia, scaly plaques or pruritic lesions. The secretions of the anal sacs were sampled by using a lavage with 0.9 % sodium chloride solution. The samples were inoculated on Sabouraud dextrose agar with chloramphenicol (SCA) (HiMedia Laboratories Pvt. Ltd., Mumbai, India), Modified Leeming & Notman agar medium (MLNA) [19] and Modified *Candida*-Chrom agar (HIT) with Tween 40 [16] and incubated at 32 °C for 7 days. The preliminary identification of yeasts was based on both the macroscopic appearance of the colonies and the microscopic cell morphology. Each sample was stained by Gram and examined by microscopy for the presence of the typical *Malassezia* yeast cells. More detailed identification was performed according to K a n e k o et al. [17]. DNA was recovered from solitary colonies grown on MLNA at 32 °C for four days.

All phenotypically positive samples recognized as *Malassezia* yeast cells were investigated by PCR-RFLP [14]. The Internal Transcribed Spacer 2 region (ITS2) was amplified by PCR using the ITS3 (5´-GCATCGATGAAGAACG-CAGC-3´) and ITS4 (5´-TCCTCCGCTTATTGATAT-GC-3´) primers [30] (Life Technologies, California, USA). PCR was modified according to G a i t a n i s et al. [15] and performed in a total volume of 50 µl. The reaction mixture consisted of 1×concetrated PCR Buffer (Life Technologies, California, USA), 3 mmol.l^{-1} MgCl$_2$ (Life Technologies, California, USA), 15 µmol of each primer (Life Technologies, California, USA), 0.1 mmol of dNTPs (Thermo Fisher Scientific, Massachusetts, USA), 2.5 U of Taq polymerase (Life Technologies, California, USA) and 2 µl of template DNA. Five µl of the PCR products were analyzed by electrophoresis in 1.5 % agarose gel at 120 V for 1 h; the gel was stained (GelRed, Biotium Inc., California, USA) and visualized under UV light. Lengths of the amplified DNA fragments were verified using GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA) and ran simultaneously. The restriction endonucleases AluI, BanI and MspA1I (New England Biolabs, Massachusetts, USA) were used for digestion of the PCR products at 37 °C for 3 h [16] in the amount of 10 U. Restriction fragments were analyzed in 3 % GelRed stained agarose gel at 120 V for 2 h and were visualized by UV light. Lengths of the amplified DNA fragments were verified using Thermo Scientific GeneRuler Low Range DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA). Reference strains of *Malassezia* spp. (M. cuniculi CBS 11721, *M. pachydermatis* CBS 1879, *M. furfur* CBS 4162, *M. slooffiae* CBS 7956, *M. globosa* CBS 7874, *M. nana* CBS 9557, *M. sympodialis* CBS 8334,

M. equina CBS 9969, *M. caprae* CBS 10434) (CBS-KNAW Fungal Biodiversity Centre Utrecht, Netherland) were used as a positive control.

RESULTS

Of the 300 animals examined, *Malassezia* yeasts were detected in 76 dogs by cultivation and microscopical methods (Table 1). From these positive animals, a total of 84 isolates were collected, of which 79 were identified as *M. pachydermatis* (six dogs had atopic dermatitis and also otitis externa with the occurrence of *M. pachydermatis*). Four isolates were identified as *M. furfur* (two *M. furfur* samples were isolates in association with *M. pachydermatis*) and one as *M. nana*. All phenotypically positive samples were confirmed by PCR-RFLP, obtaining the same results. In our group of dogs with clinical manifestations of disease,

Table 1. Prevalence of *Malassezia* species in diseased dogs

	Total number of dogs	Number of positive dogs	Prevalence [%]
Clinical status			
Diseased	300	76	25.3
Sex			
Male	174	44	25.3
Female	126	32	25.4
Age			
< 1 year	54	13	24.1
1—10 years	220	58	26.4
> 10 years	26	5	19.2
Type of ears			
Pendulous	180	59	32.8
Erect	120	17	14.2
Diagnosis			
Otitis externa	125	45	36.0
Dermatitis	106	26	24.5
Interdigital dermatitis	55	9	16.4
Inflammation of anal sacs	14	2	14.3

the prevalence of *Malassezia* spp. was 25.3 %. The highest prevalence of *Malassezia* was determined in dogs with otitis externa (36.0 %), followed by dermatitis (24.5 %), interdigital dermatitis (16.4 %) and infected anal sacs (14.3 %). The Malasezia yeasts occurred most frequently in the group of dogs with pendulous ears (32.8 %) in comparison to dogs with erect ears (14.2 %). No differences related to gender or age were observed.

DISCUSSION

Due to the variability of the phenotypic methods for the precise identification of the pathogen, we used a genotypic method based on PCR-RFLP. This study demonstrated that RFLP applying restriction enzymes, which is one of several molecular techniques used for identification and classification of *Malassezia* yeasts, is a quick and accurate method.

From the microbiological perspective, *Malassezia* is considered to be the primary fungal pathogen in dogs [7]. Our results indicated that the prevalence of *Malassezia* spp. in diseased dogs was 25.3 %. On the other hand, N a r d o n i et al. [23] described a prevalence of 67.6 % in dermatologically diseased dogs.

Ears are susceptible to unusual local conditions — high humidity, presence of cerumen and less air circulation — which constitute an ideal environment for *Malassezia* growth. Otitis externa is not a life threatening disease but can be frustrating for both patients and owners. We detected a 36.0 % prevalence of *Malassezia* species in the external ear canals of dogs with otitis externa. C a f a r c h i a et al. [5] isolated *Malassezia* yeasts from 57.3 % of the dogs with otitis and from 28 % of the dogs without otitis externa. N a r d o n i et al. [22] detected *Malassezia* spp. in 63.4 % of the dogs with otitis. In contrast to this, S a r i e r l e r and K i r k a n [27], in their study of 234 dogs with otitis externa, found *M. pachydermatis* only in 5.12 % of the samples and C a m p b e l l et al. [7] in 17 % of normal and diseased canine ears. According to the authors, the incidence of *Malassezia* varies and also different species of these yeasts were found. In our study, we identified 79 *M. pachydermatis* isolates, four *M. furfur* isolates and one *M. nana* from all positive samples. Similarly, C a f a r c h i a et al. [6] reported in both, healthy dogs and in dogs with cutaneous lesions, a higher prevalence of *M. pachydermatis* than lipid dependent *Malassezia* species. D u a r t e et al. [12] identified

only one atypical strain of *M. furfur* isolated from a dog. Also, other results suggest that in the external ear canals of 57 dogs with chronic otitis externa, lipid-dependent *Malassezia* species were isolated in only three dogs. These species were identified as *M. furfur* and *M. obtuse*, but showed atypical assimilation patterns [10]. N a r d o n i et al. [22] isolated only *M. pachydermatis* in their study of 41 dogs.

In our study, we recorded the difference in occurrence of *Malassezia* yeasts in pendulous ears compared to erect ears. We suspected that the type of ears (pendulous or erect) may influences the prevalence of *Malassezia* occurrence. K u m a r et al. [18] reported that the percentage of dogs with long pendulous ears and otitis externa was similar to the percentage of dogs with erect ears and medium hair on the ears, but C a f a r c h i a et al. [5] reported that dogs with pendulous ears showed a higher incidence of infection than dogs with erect ears.

The most frequent conditions that may contribute to *Malassezia* overgrowth on skin are: hormonal imbalance, keratinization defects, excessive production of sebum, bacterial infections and hypersensitivity processes [28]. Other important causes of *Malassezia* occurrence in our patients were also dermatitis (24.5 %) and interdigital dermatitis (16.4 %). N a r d o n i et al. [22] considered the interdigital space to be the place with the highest *Malassezia* prevalence, but it was not confirmed in our results.

Our results show that 14.3 % of dogs with inflammation of the anal sacs were positive for *M. pachydermatis*. Cytological quantification of *Malassezia* in the anal sac contents of healthy dogs revealed a low occurrence of yeasts, with only 12.5 % in the examined dogs and 10 % of the anal sacs, demonstrating the presence of *Malassezia* yeasts [25].

Some authors described a predisposition of *Malassezia* infection to be dependent upon the age of dogs [8, 21]. We did not find a difference between the occurrence of *Malassezia* spp. and the age of the diseased dogs, which was similar to the results of P l a n t et al. [26] and N o b r e et al. [24].

CONCLUSIONS

In conclusion, all isolated *Malassezia* species in our study were identified both phenotypically and genotypically as *M. pachydermatis*, except for four isolates that were identified as *M. furfur* and one isolate, which was identified as *M. nana*. No other species of *Malassezia* was found.

The highest prevalence of *Malassezia* was detected in dogs with otitis externa. Our study confirmed that *Malassezia* remains the most prevalent yeast found in the dog and that the occurrence of other species is infrequent.

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