Isolation and Differentiation of Malassezia Species Isolated from Healthy and Affected Small Animals, Ear and Skin

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Abstract: The genus Malassezia is part of the indigenous mycoflora of the mucosa and skin of healthy cats and dogs. Otherwise, it is able to survive in the environment only for short periods. In addition, their importance as emergent pathogens in humans is increasing because they have been identified as causative agents of sepsis in immunocompromised patients and neonates receiving parenteral lipid alimentation. There is only scanty information about the epidemiology and ecology of Malassezia species.

Key words: Malassezia • Animal health • Isolation • Physical characteristics

INTRODUCTION

The genus Malassezia is part of the indigenous mycoflora of the mucosa and skin of healthy cats and dogs [1]. Otherwise, it is able to survive in the environment only for short periods [2].

Whereas such yeasts are commensal microorganisms, particularly dermatitis and otitis in dogs and cats [1, 2]. In addition, their importance as emergent pathogens in humans is increasing because they have been identified as causative agents of sepsis in immunocompromised patients and neonates receiving parenteral lipid alimentation [3].

Otitis externa and superficial dermatitis are prevalent diseases in dogs and cats (9) that are frequently associated with a large number of M. pachydermatis [2].

Several studies revealed the frequency of isolation of this species from these locations is 50-83% for dogs and cats [1, 3, 4-6].

Some studies shown that M. pachydermatis is found in 57% of dog ears affected by otitis externa and in 17% of those that are clinically normal [7].

Only little is known about the frequency of the other Malassezia species in animals (8). Isolation of M. sympodialis [1, 7] and M. globosa [7] from feline skin and ear, M. furfur from feline external ear canals [5] or M. furfur and M. obtusa from otitis externa of canine [7] were recently reported.

Therefore, there is only scanty information about the epidemiology and ecology of Malassezia species [7].

Several differentiating systems, based on biochemical and physiological differences and suitable for routine diagnosis, have been published.

The purpose of the present study is to make use of these metabolic differences and assay techniques in the routine differentiation of veterinary isolates, to validate the methods and possibly gain insight into the epidemiology and ecology of the species identified [7].

MATERIALS AND METHODS

Samples: A total of 152 dog and cat specimens, including the control and case groups, submitted during November 2005 through March 2007 were examined for the occurrence of Malassezia. The sampling was accomplished from animals referred to the Small Animal Clinic of faculty of Veterinary Medicine, University of Tehran and then the samples were transferred to the Mycology Research Center of university of Tehran.

From the total of 152 samples, there were 90 samples in disease group and 62 samples in healthy group.

From the total of 90 samples (46 male and 44 female) in disease group, there were 67 (74.44%) and 26 (28.88%) from animals with otitis externa and seborrheic dermatitis, respectively.
From the total of 62 samples (29 male and 33 female) in healthy control group, there were 59 (90.32%) and 29 (9.67%) from the ear and skin animals, respectively. A questionnaire was used to getting informative data about history of each animal.

**Collection and Culture of Samples:** The collection technique used for skin and ear canal were scraping with a scalpel and cotton-tipped swab, respectively. Direct microscopy with KOH 20% and methylene blue staining were carried out in both of case and control samples.

All slides were examined at 1,000X (high power under oil immersion) magnification. When a minimum number of yeast cells per oil immersion field(oif) was exceeded (>1 yeasts/oif on the skin, > 5 yeasts/oif in ear canal exudates) (18), excessive colonization by the organism(i.e., infection) was diagnosed and these dogs/cats were assigned to the disease group.

The isolates were cultured on Sabouraud glucose agar and modified Dixon agar, containing 0.05% chloramphenicol (Merck, Darmstadt, Germany) and 0.05% cyclohexamiede (Sigma, St Louis, MO, USA). They were kept at 31°C for 7 days.

**Physiological Characteristics**

**Catalase Reaction:** The presence of catalase was determined by using a drop of hydrogen peroxide (3% solution) and production of gas bubbles was considered as a positive reaction (3). *M. restricta* is the only Malassezia species that exhibit no catalase activity (1). *M. pachydermatis* isolates have variable reaction (6).

**Demonstration of M. pachydermatis:** As *M. pachydermatis* is the only Malassezia species that is not obligatory lipid dependent (1,7), the yeast isolated on mDixon agar were smeared with a sterile swab on Sabouraud glucose agar(SGA) plus 0.05% chloramphenicol and 0.05% cyclohexamiede devoid of lipids. Incubation was performed at 31°C for one week.

**Tween Assimilation Test:** According to the method reported by Guillot et al. [1, 7], ability to utilize different Tween compounds as a unique lipid supplement by Malassezia species was evaluated. Briefly, yeast suspension (2×10^8 to 3×10^9 cfu/ml) was made in 1 ml sterilized distilled water and poured into plate containing SGA with 0.05% chloramphenicol and 0.05% cyclohexamiede that cooled to about 50°C.The inoculum was then spread evenly. After solidification of each plate, four holes were made by means of a 2 mm diameter punch and filled with 5 µl of Tween 20, 40, 60 and 80, respectively. The plates were incubated for one week at 31°C. Utilization of Tweens was assessed by the degree of growth and/or reaction (precipitation) of the lipophilic yeasts around the wells [7].

**Splitting of Esculin:** To improve the differential diagnosis of different species of Malassezia as published previously [8, 9], the β-glucosidase activity was assayed using esculin agar tube (Merck, Darmstadt, Germany). A loop of fresh (2-to-3-day-old) yeasts was deeply inoculated into the agar and incubated at 31°C for 5 days. The splitting of esculin into esculentin and glucose is revealed by darkening of the medium, with liberation of soluble ferric salt incorporated in the medium. Listeria monocytogenes and Streptococcus agalactiae served as positive and negative control, respectively.

Significant brown staining of more than a third of the medium is considered demonstrative of *M. sympodialis* and *M. obtusa* [8, 10]. *M. furfur* causes weak staining. *M. pachydermatis* has a variable reaction [10]. The others of Malassezia species are negative.

In order to differentiation of *M. sympodialis* and *M. obtusa*, they were incubated at 40°C for one week [10, 11].

**Pigment Induction Medium (P-agar):** The medium for induction of pigment synthesis was used as fallows [12, 13], per 1 liter medium, it consisted of 30 ml Tween 80 and 20 gr agar. After sterilization and cooling at 50°C, sterile filtered L-Trp (Sigma, St Louis, MO, USA) at a concentration of 15mmol was added and 6 ml each of the medium was poured into sterile Petri dishes. Suspensions of each strain were smeared on the agar medium by means of a swab. The plates were incubated for 15 days for *M. furfur* and 4 weeks for *M. pachydermatis* at 31°C, respectively.

**Statistical Analysis:** Quantitative data were analyzed by the group t-test. The data of the diseased and healthy controls were analized using chi-square test. A P-value of < 0.05 was considered significant.

**RESULTS**

From the disease and control groups, 51% and 47% were male, respectively; and so dogs and cats of either sex were found equally susceptible to these yeasts, which is similar to the findings of Kumar et al. [5] who reported from 83 cases of Malassezia otitis, 53.01% and 46.99% were male and female, respectively. Sharma et al. [2, 5] have also documented similar findings.
The highest percentage of animals having Malassezia species in both of groups were seen in 1-5 years age group with Terrier as the most frequently diseased breed.

In healthy group, roll smear cytology and cultural results were positive in 27.41% and 19.35% of the samples, respectively; whereas in disease group, they were positive about 55.55% and 40% of the samples.

Of the healthy group, 12(19.35%) and 5(8.06%) were positive in the ear and skin samples in culture media (SGA/ mDixon), respectively.

Of the disease group, 27(30%) of the ear samples and 10(11.11%) of the skin samples were positive in culture media (SGA/ mDixon).

The rest of samples of both groups were negative by the reasons of:

- Overgrown with saprophytic molds before yeast colonies had grown
- Bacterial populations were overcome yeast populations

Of the healthy group, 9(14.5%) and 11(17.74%) of 62 cultured samples were positive on SGA and mDixon agar, respectively, whereas of the disease group, 31(34.44%) and 35(38.88%) of 90 samples were positive on SGA and mDixon agar, respectively.

Using the combination identification system, in disease group with culture positive, the most commonly isolated species were M. pachydermatis (86.48%), followed by M. sympodialis (40.54%), M. furfur (16.21%), M. obtusa (8.10%), M. globosa (2.70%) and M. restricta (2.70%).

In healthy group with culture positive, the commonly isolated species were M. pachydermatis (83.33%), followed by M. sympodialis (50%) and M. obtusa (8.33%)

**DISCUSSION**

Malassezia yeasts are principally isolated from the skin of a variety of mammals and birds and are seldom recovered from the environment [2, 5, 10, 13].

Malassezia-associated skin diseases, including tinea versicolor, folliculitis and seborrheic dermatitis, is one of the major classes of superficial cutaneous mycotic infections caused by several different Malassezia spp. [1].

The importance of M. pachydermatis in dogs has been extensively reported [3]. This species can play an important role in chronic dermatitis and otitis externa in carnivores, especially in dogs. This yeast has an opportunistic nature and it may become pathogenic with any alteration in the skin surface microclimate or in host defense. Canine and feline otitis externa and seborrheic dermatitis are frequently associated with large numbers of M. pachydermatis [3].

In this study, the prevalence of M. pachydermatis in healthy ears and otitis externa was 83.3 and 70.27%, respectively.

Generally, there is no agreement between various studies about the incidence of the yeast either in healthy (15-49%) or in otitic (2-80%) ears. Many authors have reported as high as 50% occurrence of M. pachydermatis in otitic ear canals [9]; for example, Rocha et al. (2005) reported that M. pachydermatis was identified cytologically and culturally in 57.53% and 30% of samples from the ears of dogs with otitis and healthy dogs, respectively [1, 7].

Greene et al. (1998) showed that M. pachydermatis has been identified in 50-83% of dogs with otitis externa and in 19% of cats with otitis externa [1, 7].

The prevalence rate of M. pachydermatis associated with clinical cases of otitis externa reported here (70.27%) is almost similar to those reported by Gustafson [5, 9], Fraser [5, 13], Nobre et al. [5, 12], Wallmann [5, 14], Kumar et al. [5] and Kiss et al. [5, 15] as more than 70%.

Also, in our study, M. pachydermatis was isolated from 66.66% and 21.62% of healthy skin and chronic (seborrheic) dermatitis, respectively.

In this study, the results of the prevalence of M. pachydermatis in healthy ears and otitis externa are not significant; and the high percentage of this species in healthy group (ear and skin) as compared with disease group can be caused of these reasons:

- The numbers of studied animals (the numbers of the disease group as compared with healthy group).
- Overgrown with saprophytic molds before yeast colonies had grown.
- Bacterial populations were overcome yeast populations.

Classically, lipid-dependent species were related to human skin only, but it is now known that the skin and mucosa of different animals also can be colonized by lipid-dependent species in addition to M. pachydermatis [3].

The role of lipid-dependent species in human skin is well documented.

They are commensal yeast that may become etiological agents of cutaneous and systemic diseases. However, very little is known about their role animal skin [3].

Recently, lipid-dependent species have been also reported in mixed cultures from canine and feline samples,
using identification techniques such as the Tween diffusion test, the splitting of esculin and the pigment synthesis [16].

_M. sympodialis_, _M. globosa_, _M. furfur_ have been described to colonize the skin and mucosa of healthy cats [1, 2, 3, 10]. _M. furfur_ and _M. sympodialis_ also have reported from canine samples [3].

Also, it is seemed that one samples may be contain the more of one species of Malassezia: so, for obtain of this aim, it should be used from different identification techniques.

For this reason, in this study, we used from above techniques.

In this manner, we can isolate mixed cultures from 41.66% and 54.05% of control and diseased groups, respectively.

The problems encountered in recent studies have been the changing rate of culture with reporters because culture–based methods can be biased by different growth rates and culture requirements in different species and _M. restricta_ may not be detected in samples in which a catalase reaction-positive Malassezia species is mixed. On the other hand, it is not sufficient the only of the negative response of catalase reaction for _M. restricta_; because the _M. pachydermatis_ strains can exhibit variable reaction [17].

In this study, we can identify only one isolate of _M. restricta_; so, in order to resolve these problems and examine the exact distribution of these microorganisms in the skin/ear of animal/human, therefore, it is suggested to use of molecular methods at the future.

REFERENCES