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Identification and distribution of *Malassezia* spp on healthy horses skin in the north of Iran

Ayatollah Nasrolahi Omran

Associate professor, Department of Medical Mycology, Faculty of Medicine, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran,

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ABSTRACT

The genus *Malassezia* consist of lipophilic yeasts are known to be as component of the normal microflora of human skin and many mammals and birds. The purpose of this study was determinate the distribution of *Malassezia sp.* on the horses' skin in the in the north of Iran and identification of them according to horses' sex, age, breed and living geographically area. During the 15-month period, sampling was carried out using the scraping methods from different areas of skin; 256horse. A part of samples surveyed microscopically with methylene blue stain methods. All samples were inoculated onto sabouraud glucose agar media supplement with olive oil. The identification to lipid – dependence yeasts was based on the ability to use certain Tweens. The cremophore E1 assimilation test and splitting of Esculin were used as additional key characters. Other tests such as the catalase reaction and the morphological characteristics on SGA was administered. The prevalence of *Malassezia spp.* in the studied horses was 33.98%. *M.pachydermatis* was only isolated in 12 horse whereas lipid-dependent species isolated in 75 horse. The most frequently isolated *Malassezia spp.* was in groin with 24.27%, and the least of it, dorsum 15.54%. The most frequently isolated species were *M. globosa* (22.33%) followed by *M. fur fur* (18.45%), *M. restricta* (11.59%), *M. pachydermatis* (11.65%) ,*M.obtusa* (11.65%), *M. sympodialis* (11.65%) and *M.sloffiae* (10.69%). Distribution of *Malassezia species* in the healthy body have been investigated using culture-based and biochemistry techniques, and variable results have been reported from different geographical regions.

1. Introduction

Malassezia species are lipophilic yeasts commonly recognized as the resident microflora of the skin of warm-blood vertebrates and birds that can become pathogenic under certain conditions (Crespo et al., 2002; Raabe and Mayser, 1998). Several exogenous and endogenous predisposing factors such as high temperature, high humidity, greasy skin, corticosteroid treatment, immunodeficiency,

impaired balance of the normal microflora of the skin, altered composition of surface lipid and previous antibiotic treatment can influence these yeasts to become pathogenic, and play an important role in chronic dermatitis and otitis external especially in Animals (Durate and Batista, 2003; Gupta et al., 2000; Harada et al., 2015; Sparber et al,2017).

*Corresponding author: Dr. Nasrolahi Omran
Phone: +9891113753429
E-mail: ayat51@yahoo.co.in

Taxonomy of these yeasts has always been a matter of controversy. The genus has recently been revised on the basis of Morphology, ultrastructure, physiology and molecular biology enlarged to twelve species. They include the three former species *Malassezia furfur* (Robin) Baillon 1889; *Malassezia pachydermatis* (Weidman) Dodge 1935, *Malassezia sympodialis* (Gueho) Simmons 1990 and new taxa *Malassezia globosa*, *Malassezia obtusa*, *Malassezia restricta* and *Malassezia slooffiae* (Gueho and Guillot, 1996; Weiss et al., 2000). Afterwards, seven new lipid – dependent species belonging to the genus *Malassezia* have been described or proposed. From these seven species, *Malassezia dermatis* (Sugita and Takashemia, 2002), *Malassezia japonica* (Sugita and Takashemia, 2003), *Malassezia yamatoensis* (Sugita and Tajima, 2004), *Malassezia nana Hirai* (Hirai and Kano, 2004), *Malassezia cuniculi* (Cabañes et al., 2011) and the tentatively named *Malassezia equi* (Nell and Bond, 2002), *Malassezia caprae* (Cabañes, 2007), *Malassezia psittaci* (Lorch, 2018) are genetically close to the type strain of *Malassezia spp.* and almost all of them also have some common morphological and physiological characteristics.

Malassezia Pachydermatis is the only species in the genus that does not require lipid supplementation for development in culture medium. The remainder species are lipid-dependent because they require long, chain fatty acid for in vitro growth (Guillot and Bond, 1999). The occurrence of different species of *Malassezia* has been reported from domestic and ruminants Animals (Castellá et al., 2005; Crespo et al., 2002). Quantifying the number of *Malassezia* recovered has been used as an indirect evidence to support a role for the organisms in the pathogenesis of certain skin disorders (Gueho and Guillot, 1998; Nardoni et al., 2004). The molecular characterization of *Malassezia spp.* isolates from animals and humans has not been thoroughly studied (Cabañes et al., 2007).

Laterly, several new lipid-dependent species belonging to the genus *Malassezia* have been described Intra-species variations in DNA pattern of *Malassezia* isolates and the presence of specific genetic types in animals or humans were observed. The validation of new species was supported by analysis of the D1/D2 regions

of the 26S rRNA gene and the ITS-5.8S rRNA gene sequences. DNA characterization by D1/D2 26S rRNA gene and internal transcribed spacer (ITS)-5.8S rRNA gene sequencing analysis of lipid-dependent strains from different animal species described and illustrated (Cabañes et al., 2005; Duarte et al., 2009; Duarte, 2010). The study focus on scarcely investigated epidemiological aspects of *Malassezia spp.* in swine (Nardoni et al., 2009). The purpose of this study was to determinate the prevalence of *Malassezia spp.* on the horses' skin in the Golestan province and their identification based on horses sex, breed, age and living geographical area.

2. Materials and Methods

During the 15 month period, sampling was carried out using the sterile moquet (kind of carpet) and the scraping methods from horses perineum, ear, axilla, groin and dorsum. A part of samples was surveyed microscopically with methylene blue. All samples were inoculated onto the Sabouraud glucose agar media (SGA) supplemented with olive oil. All media contained 150 mg of chloramphenicol and 1 gr of cycloheximide. Plates were incubated at 30 °C and were studied in 3, 5, 7 and 10 days intervals. When the growth to *Malassezia* colony was observed, the colonies sub cultured on SGA to determine their lipid – dependence. *Malassezia pachydermatis* was identified by gross colony and microscopic morphology and by its ability to grow on SGA. After 7 days of incubation at 30 °C on SGA, the typical lipid-dependent colonies were cream – colored, smooth, small and slightly folded with a convex elevation. The identification of lipid – dependent yeasts was based on the ability to use certain polyoxethylene sorbitan esters (Tween 20, 40, 60 and 80). Then the identification of species was described by Gueho et al., (Gueho and Guillot, 1996) and the Tween diffusion test was proposed by Guillot et al (Guillot, J. Gueho, E., Dupont, B., 1996). The Cremophore El assimilation test and splitting of Esculin were used as additional key characters. Other tests such as the catalase reaction and the morphological characteristics on SGA were administered (Mayser and Gueho, 1997) (The statistical test used for statistical analysis was chi-square test.

3. Results

Our results showed that %50.78 (130 cases) of horses were male and the rest were mare 49.22% (126 case). Those horses 90 case (35.15%) were Turkoman breed and rest were 17 cases (30.90%) blood' breed Turkmen horse, 69 cases (20.95%) of the horses thoroughbreds and 20 cases (7. 81%) were of unknown breed. The average age of the horses was 7 years, most frequent of which were in the age group of 2-8years, 115 cases (44.42%) and the rest were a groups of under 2 years, 50 cases (19.53%), the group of over 8 years contained 91 cases (35.55%). From 256 horses that were studied 50 cases (19.53%) were male and 37 cases (14.45%) mare had *Malassezia* positive culture. The highest prevalence of *Malassezia* was seen at the age group of 2-8 years in 55 cases (21.48%) and in the Turkmonan breed in 37 cases (14.45%), the lowest result was at age group under 2 years in 12 case (4. 69%).The significant correlation was not observed between the horses sex, breed, living geographical area and prevalence of *Malassezia spp.* whereas a significant correlation seemed to exist between horses' age and prevalence of *Malassezia*. Typical *Malassezia* cells were not observed in cytological examinations. *Malassezia spp.* positive cultures were obtained from 87 horses. Some cultures overgrown by contaminants or

otherwise could not be sub-cultured, but 103 purified sub cultured culture were obtained. The prevalence of *Malassezia spp.* in the studied horses was 33.98%. The species *Malassezia pachydermatis* was only isolated in 12 horses whereas lipid-dependent species isolated in 75 horses. The distribution of *Malassezia spp.* in different skin areas and isolated *Malassezia spp.* is detailed in table 1. The most frequently isolated *Malassezia spp.* was in groin with 25 cases (24.27%), followed by perineum 23 cases (22.33%), ear 22 cases (21.36%), axilla 17 cases (16.5%) and dorsum 16 cases (15.54%). The most frequently isolated species were *M. globosa* with 23 case (22.33%) followed by *M. furfur* 19 cases (18.45%), *M. restricta* 14 cases (11.59%), *M. pachydermatis* 12 cases (11.65%), *M. obtusa* 12 cases (11.65%), *M. sympodialis* 12 cases (11.65%) and *M. slooffiae* 11 cases (10.69%). *M. pachydermatis* was isolated from the anus, ear and dorsum. *M. fur fur* was isolated from all sampling skin areas in the horses. *M. sympodialis* was isolated from all sampling skin areas except anus. *M. obtusa* was isolated from the perineum, ear. *M. globosa* was isolated from all sampling skin areas expect ear *M. slooffiae* was isolated from groin, perineum and ear. *M. restricta* was isolated from axillia, groin and ear.

Table 1. Distribution of *Malassezia spp* in different skin areas and isolated *Malassezia* species. N= number P=percent

Site <i>Malassezia</i>	Axilla		Groin		Dorsum		Perineum		Ear		Total	
	P	N	P	N	P	N	P	N	P	N	P	N
<i>M. fur fur</i>	11.7	2	20	5	43.75	7	13.05	3	9.09	2	18.45	19
<i>M.pachydermatis</i>	-	-	4	1	12.5	2	8.7	2	31.82	7	11.65	12
<i>M. sympodialis</i>	23.5	4	20	5	6.25	1	-	-	9.09	2	11.65	12
<i>M. obtusa</i>	-	-	-	-	-	-	30.43	7	22.73	5	11.65	12
<i>M. globosa</i>	35.3	6	16	4	37.5	6	30.43	7	-	-	22.33	23
<i>M. slooffiae</i>	-	-	20	5	-	-	17.39	4	9.09	2	10.68	11
<i>M. restricta</i>	29.4	5	20	5	-	-	-	-	18.18	4	13.59	14
Total	100	17	100	25	100	16	100	23	100	22	100	103

Yeasts of the genus *Malassezia sp.* inhabit in the skin of variety of mammals and birds. These yeasts are capable to act as opportunistic pathogens both in man and animals (Gueho and Guillot, 1998; Weiss et al., 2000) Classically, lipid – dependent species were related to human skin only but it is known that the skin to different animals can also be colonized by the lipid -dependent species in addition to *M. pachydermatis* (Crespo et al., 2002; Guillot and Bond, 1999). The role of the lipid -dependent species in human skin is well documented, but very little is known about their role in horses and other animals; therefore, more studies would be made to determine their role in animals (Gupta et al., 2000; Weiss, 2000). In the research conducted by Azarvandi with purpose to isolate and identify yeast flora in the caudal reproductive tract in healthy female horses. Yeast colonies were isolated from 60 (39.7%) of the 151 horses. The isolated yeasts belonged to nine genera, and included *Candida spp.* (53.2%), *Cryptococcus spp.* (12.2%), *Saccharomyces spp.* (10.5%), *Geotrichum spp.* (8.0%), *Rhodotorula spp.* (7.1%), *Malassezia spp.* (3.7%), *Trichosporon spp.* (2.6%), *Kluyveromyces spp.* (2.6%) and *Sporothrix spp.* (0.2%). *Candida krusei* (43.1%) was the most frequent *Candida species* isolated (Azarvandi A,2017).

The prevalence of *Malassezia spp* in horses (33.98%) in our work was smaller than what was reported by Crespo (Crespo et al., 2002; Crespo et al., 2002) for these animals (60%).The different in the results of our study and that of Crespo in the prevalence of *Malassezia spp* may also reflect the different horses sex, breed, studied geographically areas and sampling method (Crespo et al., 2002a).A higher prevalence of positive culture of *Malassezia spp.* was observed in healthy adult horses than in healthy younger and older horses. Hormonal differences could be responsible for the higher prevalence of the yeasts in the male studied. No significant correlation was observed between the horses' sex, breed, living geographical area and prevalence of *Malassezia spp.* whereas a significant correlation existed between horses age and prevalence of *Malassezia spp.* On the other hand, Crespo study (Crespo et al., 2002a) did not argued these variables.

In our study the prevalence of lipid – dependent species (88.75%) was much greater than that of *M. pachydermatis* (11.43%). The result's of our study are in concordant with Crespo (89.19%, 10.8%) study (Crespo et al.,2002). Our results confirmed that the most frequently isolated species were *M. globosa* (22.33%) and *M. fur fur* (18.95%), as the second isolated in importance. The common prevalence of these species among horses are in a concordance with the findings of Crespo which held that these yeasts were the most frequently isolated species. Recent studies have shown that horses of different body sites have different *Malassezia spp.* indicated conformity with Crespo findings (Crespo et al., 2002).

To our knowledge, this is the first report of isolation of the lipid -dependent species *M. sympodialis* from horses ear, dorsum and groin. Based on the finding of this study, lipid -dependent species seem to be more frequent in horses. The findings confirm healthy horses in addition to the fact that non lipid – dependent can colonize *Malassezia lipid – dependent sp.* However, it is still unclear what the role of the lipid – dependent species in the horses skin is. Similarly, no kind of dermatitis caused by lipid dependent species in those animals has been reported (Hirai,A., Kano,R, 2004).One of the main causes for the difficulty of characteristic of lipid – dependent species is the lack of suitable methods for the isolation and preservation of those yeasts to allow them to be studied. In direction, identification of species by biochemical and physiological test is unavoidable. Speed, accuracy, to cheap and to repeat are preferences of these tests (Gueho and Guillot,1996; Mayser and Gueho,1997).

The presence of *M. restricta* was proved when the isolates failed to demonstrate catalase activity, these species being the only *Malassezia* that lacked catalase (Gueho and Guillot, 1996; Guillot, 1996). *M.furfur*, *M. sympodialis* and *M. slooffiae* are physiologically very similar and ambiguity regarding correct identification of these species on the basis of test for the utilization of tween compounds in the sample media (Gueho and Guillot,1996; Guillot et al.,1996). The cremophor EL contains castor oil and ricinoleic acid and is used as an additional key character for identification of the species *M. furfur*, *M.slooffiae* and *M.sympodialis* (Mayser and Gueho, 1997). Microscopic morphological

findings were necessary for the identification of *M. globosa* and *M. obtusa* characterized by *M. globosa* cylindrical cells, respectively (Duarte and Hamdan, 2010; Guillot et al., 1996). Complementary test, including molecular techniques would be required to differentiate atypical lipid-dependent isolates (Gupta et al., 2000). The significant prevalence of *Malassezia* spp. in healthy horses in this and previous (Crespo) studies indicate that it is a member of the normal microbiota of these animals.

In the research conducted by zia for detection and species-level identification of the *Malassezia* yeasts in domestic animals and aquatic birds (471 animals) by PCR-RFLP. *Malassezia* were detected at 15.46% in horses. Eighty colonies of 6 species were isolated *M. globosa* 41.25%, *M. furfur* 22.5%, *M. restricta* 15%, *M. sympodialis* 15%, *M. pachydermatis* 5% and *M. slooffiae* 1.25%. Therefore, different lipophilic *Malassezia* spp. are found in a wide diversity of animals and aquatic birds. PCR-RFLP is a suitable technique for identification of different *Malassezia* spp. (zia, 2015).

In the research conducted by Aldrovani, on characterize genotypically *Malassezia* spp. isolated from the external ear canal of healthy horses (Fifty-five). It was surprising that *M. nana* represented over 80% of the strains and no *M. equina* was isolated in this study, differing from what was expected (Aldrovani, 2017).

In the research conducted by White, *Malassezia dermatitis* has been observed in horses in intertriginous areas such as the udder and prepuce; the species of yeast was not confirmed. The samples (Eleven healthy horses) were examined cytologically and were cultured on modified SDA. The DNA from yeast colonies grown on the agar was extracted, and samples were assayed using fungal generic PCR analysis. Of 44 attempts at culture, 5 yielded a species identified as *Malassezia equi*, and 2 yielded *M. slooffiae* (White, 2006).

These findings may help to elucidate the epidemiology of disease caused by *Malassezia* spp. in animals. Thus, the predominant genetic type was observed in isolates recovered from different anatomical locations in all horses was colonized by more than one type of *Malassezia* spp. and different genetic types were detected in the same body site. It is not known whether healthy mares or male horses can be carriers of this yeast in these body areas. *Malassezia* spp.

are present in the intermammary region in healthy mares and the preputial fossa in healthy geldings.

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