Original Research

The Prevalence of Malassezia yeasts in patients with seborrheic dermatitis by PCR-RFLP method in Isfahan, Iran

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Abstract

Introduction: Malassezia spp. are lipophilic basidiomycetes that are the microbial members of the flora of most warm-blooded animals. The natural habitat of these lipophilic yeasts is the horny part of human and animal skin. Malassezia population densities in infected lesions are generally higher than in healthy skin, and yeast proliferation appears to be the first step in developing Malassezia dermatitis. Under certain conditions, these yeasts can cause diseases such as Pityriasis versicolor and seborrheic dermatitis, and even cause systemic infections. The aim of this study was to investigate the prevalence of Malassezia in Isfahan, Iran.

Material and methods: In this descriptive cross-sectional study in Isfahan, 100 people (70 men and 30 women) with seborrheic dermatitis were studied. Sampling was performed by chipping from dandruff and behind the ears. The samples were studied microscopically. After culturing and extracting DNA from the colonies, Malassezia spp. were identified by PCR-RFLP method.

Results: Based on the results, the total number of yeast cells in the samples of patients with seborrheic dermatitis was 53 (53%) and also 38 (38%) of yeast colonies grew. Based on PCR-RFLP results, five species including *Malassezia globosa, Malassezia furfur, Malassezia restricta, Malassezia sympodialis and Malassezia slofiae* were identified.

Conclusion: Based on PCR-RFLP analysis in healthy control, seborrheic dermatitis and Pityriasis versicolor in patients referred to laboratories in Isfahan, *Malassezia globosa* was the most common isolated species among the five isolated species.

Keywords: Malassezia spp, Pityriasis versicolor, Seborrheic dermatitis, MLNA culture medium, PCR-RFLP.

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Introduction

Malassezia belong to the basidiomycota genus, heterobasidiomycete, and Cryptococcus family (1, 2). Malassezia reproduces sexually and germination is unipolar. This yeast is the normal flora of most warm-blooded vertebrates, including humans and a variety of mammals and birds. Because Malassezia is lipophilic, its natural analgesic is oily skin (3). Malassezia species are highly prone to fat as a substrate, a feature used to identify them.In laboratory culture media, they are rarely isolated and special media are required to separate them.So far, 14 species of Malassezia have been identified based on morphological, extra-structural, physiological and genetic studies (4. (This yeast is the normal flora of most warm-blooded vertebrates, including humans and a variety of mammals and birds. However, the population density of Malassezia in infected skin lesions is generally higher than that of healthy skin, and yeast proliferation appears to be the first step in developing Malassezia dermatitis (5, 6).This yeast is involved in the development of diseases such as pityriasis versicolor, seborrheic dermatitis, atopic dermatitis, folliculitis and psoriasis.(7, 8).

Yeast cell coatings include distinct layers such as outer coatings, cell walls, plasma membranes, and special building blocks identified by electron micrographs. Seborrheic dermatitis is an inflammation of the skin, chronic, inflamed and dandruff with an increase in sebum in the head, face and upper torso. The disease can affect infants, adults and adults of all races and genders. The prevalence of seborrheic dermatitis varies from 1 to 5% in competent immunocompromised individuals but increases in immunocompromised individuals. Factors such as oily skin, corticosteroid therapy, and immunodeficiency increase the susceptibility to Malassezia-related diseases. High ambient temperature and high relative humidity are effective in causing diseases caused by Malassezia. Due to the possibility of blood infection by this yeast in patients receiving lipid drugs in the hospital, their importance as harmful pathogens in humans is increasing (9, 10).

Although the pathophysiology of seborrheic dermatitis is not fully understood, the association of the disease with the proliferation of Malassezia species and the clinical response of the disease to antifungal agents (such as ketoconazole, cyclopirox) has led many researchers and physicians to believe in a central role in the pathogenesis of seborrheic dermatitis. At present, species identification is based on morphological, extra-structural, physiological and molecular studies, and based on this, 14 Malassezia species have been identified. Since the morphology of different species is almost the same and it is also difficult to isolate and maintain some species such as *M. restricta* and *M. globosa*, there are ambiguities in interpreting the results of phenotypic methods. They lack sufficient differentiating power and cannot clearly identify newly identified species (11, 12). Therefore, researchers prefer to use molecular methods to identify and differentiate species. Therefore, due to the extraordinary importance of these yeasts, the use of molecular methods and the need for additional studies to identify Malassezia species is necessary. The aim of this study was to evaluate Malassezia species among patients with seborrheic dermatitis by PCR-RFLP method in Isfahan.

Materials and Methods

This descriptive cross-sectional sampling study was conducted in Isfahan. In this study, 100 patients with seborrheic dermatitis were studied. The subjects were in the age range of 14-20 years. Sampling was performed from dandruff areas behind the ears.

Then, in the mycology laboratory of Islamic Azad University, Falavarjan branch, Isfahan, Iran microscopic examination of the samples was performed by methylene blue and wet slide staining.

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Due to not receiving the code of ethics from the university, samples from medical laboratory were observed after observing ethical points and maintaining privacy, including without the patients' names.

Identification of Malassezia species by RFLP - PCR molecular method

After culturing the samples in modified Leeming-Notman Agar (MLNA) and incubating at 32 ^{0} C for 14-7 days to determine Malassezia species by PCR-RFLP method, DNA extraction was performed by phenol-chloroform method (13).

To identify Malassezia species isolated by molecular polymerase chain reaction (PCR) and Mal F and Mal R primers related to 28SrDNA region including 5´-TAA CAA GGA TTC CCC TAG TA-3´ (Forward primer)and 5´-ATT ACG CCA GCA TCC TAA G-3´ (Reverse primer) and DNA of grown colonies were used (14).

Materials used in this experiment were 5 µl of template DNA, 5 µl of 10 X buffer, 0.5 µl of dNTP with a concentration of 2 mM, 0.25 µl of Taq polymerase enzyme, 0.5 µl of each reciprocating primer with a concentration of 10 pmol, 1.5 µl MgCl2 with a concentration of 350 mM and the final volume with 50 µl of distilled water. A 50 µl reaction was used. The thermal program for the reaction included initial denaturation at 94°C for 5 min, 30 cycles including denaturation at 94° C for 45 seconds, annealing at 55°C for one min, elongation 72°C for 45 seconds and finally the final elongation at 72 ° C for 7 min (14).

To perform Restriction Fragment Length Polymorphism (RFLP), 10 µl of PCR product in 1.5 µl of specific enzyme was used under the influence of Hin6I, 0.5 µl of Cfo1 restriction enzyme (14). 1.5% agar gel PCR and RFLP products were used to separate DNA fragments. *M.furfur* CBS9577, *M. globesia* CBS7874 and *M. sympodialis* CBS7222 were used as positive control strains in this study.

Results

A total of 160 patients including 60 patients with seborrheic dermatitis, 50 patients with PT rheumatoid arthritis and 50 healthy individuals were examined for the presence of Malassezia yeast on different levels of the body. Direct microscopic examination of 85 samples (53.13%), of which 65 samples (76.47%) were grown in MLNA medium (Table1).

After DNA extraction and PCR, Cfo1 enzyme was used to cut 28S rDNA regions and five species including *M. globosa, M. furfur, M. restricta, M. sympodialis* and *M. slofiae* were identified by RFLP. As a result, all 65 yeast colonies grown in MLNA culture medium were subjected to PCR-RFLP, which was confirmed in 56 cases of Malassezia yeast and different species were determined. The absence of specific bands in PCR or RFLP in the other 23 cases indicated that the yeasts were genus other than Malassezia (table 2 and 3).

Chi-square test showed that there was no significant difference in the distribution of Malassezia yeast species in 3 groups (healthy controls, seborrheic dermatitis and Pityriasis versicolor) (P-Value = 0.42). Chi-square test showed that there were significant differences in the total frequency distribution of different species (P-Value = 0.01). Also, the highest frequency is related to *M. globosa* with 48.2% and the lowest frequency is related to *M. slofiae* or 1.8%.

Electrophoresis of the PCR product resulting from amplification of the 28S rDNA fragment before and after digestion with the *CfoI* enzyme is shown in Figure 2.

Discussion

Lipophilic yeasts, such as species of the genus Malassezia, are known as the normal flora of the human skin and as organisms involved in the development of superficial diseases (4, 15). In this study, we decided to investigate the frequency and identification of Malassezia species in people with seborrheic dermatitis, Pityriasis versicolor and healthy control in Isfahan by PCR-RFLP method. In this study identified 5 species including *M. globosa, M.*

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furfur, M. sympodialis, M. restricta, and M. sloofiae from Pityriasis versicolor, 3 species including *M. globosa, M. furfur* and *M. restricta* from Seborrhoeic Dermatitis, 4 species including including *M. globosa, M.furfur, M. sympodialis* and *M.restricta* were isolated from healthy control.

In total, the frequency of 5 species was 48.2, 30.4, 5.4, 14.3 and 1.8%, respectively. Thus, the highest frequency was related to *M.globosa* (48.2%) and the lowest frequency was related to *M. sloofiae* (1.8%).

Due to the fact that routine laboratory diagnostic methods such as biochemical, physiological or immunological methods are associated with many problems and are not able to differentiate different species optimally, the use of molecular methods to identify Malassezia species is widely in recent years (16).

In the study of Jafari et al., The highest and lowest frequency of Malassezia were related to *M. Globosa* (38%) and *M. sloofiae* (5.3%) (17).The prevalence of *M. globosa* and *M. furfur* was higher in the study of Shokoohi et al., in Pityriasis versicolor and seborrheic dermatitis (18). In another study conducted by Hedayati et al., The highest frequency of Malassezia species was related to *M. globosa* and *M. furfur* (19), the results of which were consistent with the results of the present study. Also in 2018, Awad et al., conducted a study in Iraq entitled Phenotypic Identification and Molecular Characteristics of Malassezia Isolated from Patients with Ptriasis Versicolor in Diyala Province, and reported *M. globosa* and *M. furfur* as the most common species of Malassezia (20). Many studies in other parts of the world, including India, have shown similar results (21, 22). Therefore, *M. globosa* and *M. furfur* are the most common species of Malassezia (23). In addition, according to the results of this study and other studies, and despite culture problems related to these species, molecular methods such as PCR-RFLP can be used as a very simple method. Reproducible and precise for distinguishing between Malassezia species (16, 24). Therefore, using molecular techniques, more accurate diagnosis and treatment of different species and epidemiological studies can be achieved. One of the problems in this study was obtaining the desired samples, because in many cases, patients do not face acute problems in this disease, less often see a doctor, and with the diagnosis of the disease by doctors, sometimes patients do not go to the laboratory.

Conclusion

Based on the results of PCR-RFLP analysis as a simple and practical technique to identify different species of Malassezia in patients referred to laboratories in Isfahan, *M. globosa* was the most abundant species isolated among the five species.

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Conflicts of interest

There are no conflicts of interest.

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Table 1: Frequency of Malassezia yeasts in phenotypic tests

	Healthy Controls N (%)	**Seborrhoeic Dermatitis N (%)**	**Pityriasis versicolor N (%)**
Direct test	(48)24	(51.7)31	**(60)30**
Culture on MLNA	(40)20	(36.7)22	**(46)23**

Table 2: Number of different species of Malassezia in the samples

Total N (%)	**Seborrhoeic Dermatitis N (%)**	**Pityriasis versicolor N (%)**	**Healthy Controls N (%)**	**genus**
(30.4)17	(31.59)6	(23.8)5	(37.5)6	***M. furfur***
(48.2)27	(52.63)10	(47.6)10	(43.75)7	***M. globosa***
(1.8)1	(0.00)0	(4.76)1	(0.00)0	***M. slofiae***
(14.3)8	(15.78)3	(3 .14)3	(12.5)2	***M. restricta***
(5.4)3	(0.00)0	(9.52)2	(6.25)1	***M. sympodialis***
(100)56	(100)19	(100)21	(100)16	**total**

Table 3: The frequency of Malassezia species in the study population according to sex

	Pityriasis versicolor	**Seborrhoeic Dermatitis**	**Healthy Controls**

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genus	Female N (%)	Male N (%)	Total N (%)	Female N (%)	Male N (%)	Total N (%)	Female N (%)	Male N (%)	Total N (%)
M. furfur	(22.22)2	(25)3	(23.8)51	(40)4	(22.22)2	(31.57)6	(28.57)2	(44.44)4	(37.5)6
M. globosa	(44.44)4	(50)6	(47.61)10	(50)5	(55.55)5	(52.63)10	(57.14)4	(33.33)3	(43.75)74
M. slofiae	(0.00)0	(8.33)1	(4.76)1	(0.00)0	(0.00)0	(0.00)0	(0.00)0	(0.00)0	(0.00)0
M. restricta	(22.22)2	(8.33)1	(14.28)31	(10)1	(22.22)2	(15.78)3	(14.28)1	(11.11)1	(12.5)2
M. sympodialis	(11.11)1	(8.33)1	(9.52)2	(0.00)0	(0.00)0	(0.00)0	(0.00)0	(11.11)1	(0.00)0
total	(100)9	(100)12	(100)21	(100)10	(100)9	(100)19	(100)7	(100)9	(100)16

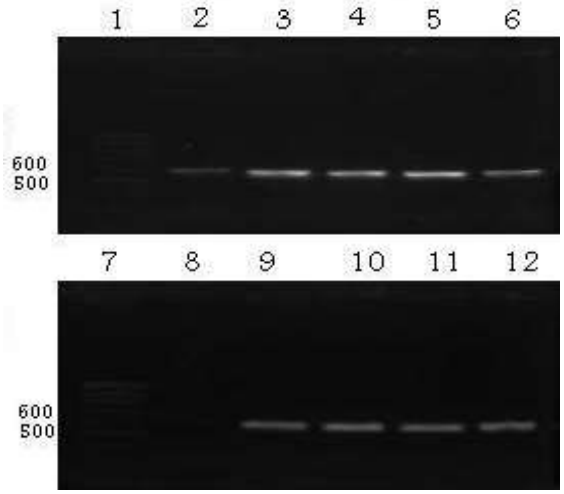
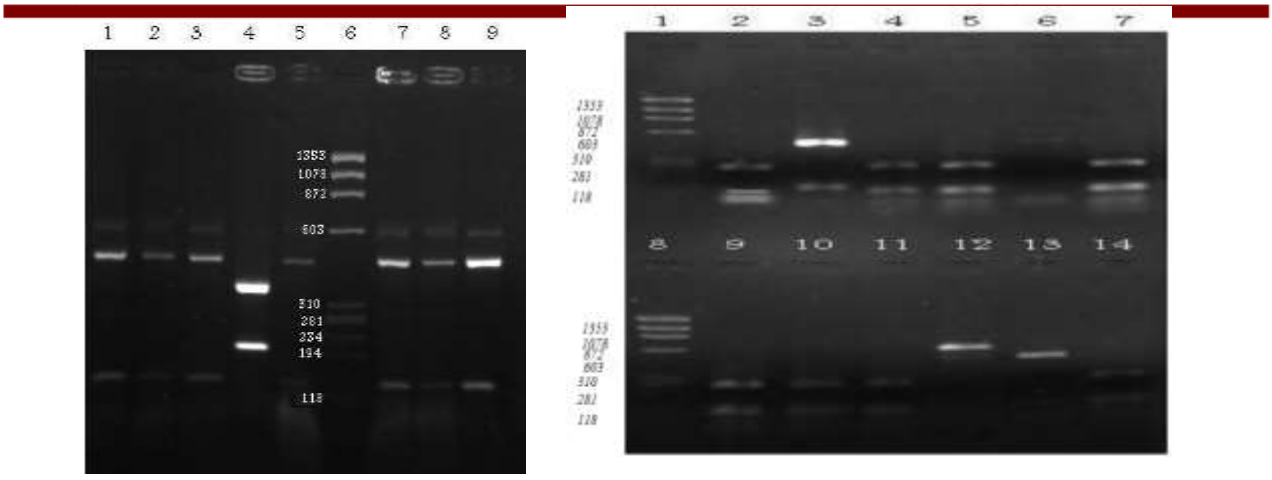


Figure 1. Electrophoresis of PCR products of amplified ribosomal DNA genomic regions. The size of all bands is between 500-600 bp (lanes 5 and 8 are positive control and negative control, respectively).

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(A) (B)

Figure 2 - Electrophoresis of digestion products with CfoI enzyme of different species of Malassezia(A: Lanes 1, 2, 3-*M. globosa*, lane 4-*M. sympodialis, lane 5-M globosa, lane 6-* Molecular marker, lane *7, 8, 9-M. globosa*. B: lane 1.Molecular marker, lane *2-M.sloofiae,* lane *3-M. globosa*, lane *4-M. furfur,* lane *5-M.Furfur* , lane *6-Negative* , lane *7-M. furfur*, lane *8* Molecular marker , lane *9-M. furfur,* lane *10-M. furfur,* lane *11-M. Furfur* , lane *12-M. restricta* , lane *13-M. globosa* lane *14-M. furfuyr***)**

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