

Molecular Identification and Prevalence of *Malassezia* Species in Pityriasis Versicolor Patients From Kashan, Iran

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Background: *Malassezia* species are lipophilic yeasts found on the skin surface of humans and other warm-blooded vertebrates. It is associated with various human diseases, especially pityriasis versicolor, which is a chronic superficial skin disorder.

Objectives: The aim of the present study was to identify *Malassezia* species isolated from patients' samples affected by pityriasis versicolor, using molecular methods in Kashan, Iran.

Patients and Methods: A total of 140 subjects, suspected of having pityriasis versicolor from Kashan, were clinically diagnosed and then confirmed by direct microscopic examination. The scraped skin specimens were inoculated in modified Dixon's medium. DNA was extracted from the colonies and PCR amplification was carried out for the 26S rDNA region. PCR products were used to further restriction fragment length polymorphism by CfoI enzyme.

Results: Direct examination was positive in 93.3% of suspected pityriasis versicolor lesions. No statistically significant difference was observed in the frequency of *Malassezia* species between women and men. The highest prevalence of tinea versicolor was seen in patients 21-30 years-of-age. No difference could be seen in the frequency of *Malassezia* species depending on the age of the patients. In total, 65% of patients with pityriasis versicolor had hyperhidrosis. The most commonly isolated *Malassezia* species in the pityriasis versicolor lesions were; *Malassezia globosa* (66%), *M. furfur* (26%), *M. restricta* (3%), *M. sympodialis* (3%), and *M. slooffiae* (2%). *Malassezia* species were mainly isolated from the neck and chest.

Conclusions: This study showed *M. globosa* to be the most common *Malassezia* species isolated from *Malassezia* skin disorders in Kashan, Iran. The PCR-RFLP method was useful in the rapid identification of the *Malassezia* species. By using these methods, the detection and identification of individual *Malassezia* species from clinical samples was substantially easier.

Keywords: *Malassezia*; Pityriasis versicolor; Molecular diagnosis

1. Background

Malassezia species are lipophilic or lipid-dependent, budding yeasts, which are part of the skin microflora of humans and other warm-blooded animals. It can become pathogenic under certain predisposing factors, such as alterations in the skin's condition and changes in the host's defenses. *Malassezia* species are associated with various human diseases, especially pityriasis versicolor (PV), which is a chronic superficial skin disorder (1). Pityriasis versicolor is a superficial fungal infection characterized by the appearance of round to oval lesions. PV is a chronic cutaneous disorder, and it can occur in any part of the body; however, it is more common in tropical, humid areas. Despite antifungal treatment, most patients with PV will face recurrence of the condition.

The disease is more common between puberty and

middle age, when there are maximum amount of *Malassezia* yeast on the skin. PV is characterized by a low to more scaly macule that can present as either hyper or hypopigmented (1), and it is usually present on the neck and upper trunk (2). Moreover, *Malassezia* species are an etiological agent of catheter-associated; fungemia, seborrheic dermatitis (3), folliculitis (1, 4), dacryolitis, and blepharitis, as well as nosocomial bloodstream infections in pediatric care units (5).

In recent years, molecular studies have changed the taxonomy of the genus *Malassezia* to a considerable extent. Sequence variability between *Malassezia* species has been documented in rRNA genes. In 1996, Gueho et al. (6), classified the genus of *Malassezia* in seven distinct species, namely; *M. furfur*, *M. pachydermatis*, *M. sympodialis*,

Implication for health policy/practice/research/medical education:

Rapid identification of *Malassezia* species is valuable, but it can often be controversial. This study will help increase understanding of the epidemiology of *Malassezia* in the central areas of Iran, which will be useful for the management of *Malassezia* disease.

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M. globosa, *M. obtusa*, *M. restricta* and *M. slooffiae*. Recently, based on DNA analysis, six new species have been introduced: *M. dermatis*, *M. nana*, *M. japonica*, and *M. yamatoensis*, *M. equine*, *M. caprae*. Of these *M. caprae*, *M. equine* and *M. nana* have only been isolated from domestic animals (6-10).

Malassezia species can be diagnosed based on their biochemical features. However, these methods do not have enough discriminatory power and therefore, cannot characterize the newly defined species. In addition, biochemical and phenotypical methods are not able to achieve an immediate diagnosis. In recent years, molecular approaches and PCR methods for the discrimination of *Malassezia* species are the most accurate (11, 12). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using restriction enzyme digestion is a simple, reliable, and cost-effective method for the differentiation of *Malassezia* species, which has been described by Mirhendi et al. (13). After amplification of the partial 26S rDNA, digestion of the PCR product with the enzyme CfoI produced the predicted species specific pattern for all species tested. Using CfoI, nine different species, including; *M. furfur*, *M. pachydermatis*, *M. globosa*, *M. obtuse*, *M. restricta*, *M. slooffiae*, *M. nana*, *M. japonica*, and *M. yamatoensis* could be distinguished, however, *M. sympodialis* and *M. dermatis* produced the same digestion pattern.

Our data offers insight into the epidemiology of PV in Iran, but it is limited to areas such as; Tehran, Ahvaz, Yazd and Mazandaran (4, 14-17). Furthermore, there is evidence suggesting geographical variations in the distribution of the species. On the other hand, the majority of these studies have identified *Malassezia* species based on biochemical and phenotypical characterization, such as assimilation of Tween, and PCR based techniques were not used.

2. Objectives

The objective of this study was to determine the *Malassezia* species which grew on culture from the skin scrapings of patients with PV in Kashan (central region of Iran), using molecular methods. There is little current information about the molecular epidemiology and ecology of *Malassezia* species available in Kashan, Iran.

3. Patients and Methods

3.1. Subjects and Collecting Samples

In total, 140 subjects with skin lesions suspected to be PV from the Shahid Beheshti Skin Clinic, during 2012, who were referred to the Fatemeh Zeidi Clinical Laboratory, Kashan, Iran, were enrolled in this study. A data collection form which included; sex, age, disease duration, and lesion area, was completed for the patients. Identification of PV on the lesions was initially clinically diagnosed after that the final diagnosis was confirmed by direct microscopic examination using the Sellotape method and

methylene blue stain for skin samples, obtained by the scraping method (18, 19).

3.2. Culture of Sample

The scraped skins were inoculated in modified Dixon medium. This medium consisted of 3.6% malt extract (Merck, Germany), 2.0% desiccated ox-bile, 1.0% Tween 40 (Sigma & Aldrich), 0.2% glycerol (Merck, Germany), 0.2% oleic acid (Merck, Germany), 0.05% chloramphenicol (Sigma, USA), 0.5% cycloheximide (Sigma, USA), and 1.2% agar. Inoculated cultures were incubated at 32°C for 1-10 days. The harvested cells were preserved at -20°C until diagnostic analysis. Suspected *Malassezia* species were identified to the level of species on the basis of the PCR-RFLP method (13).

3.3. PCR- RFLP

DNA was extracted with phenol chloroform-isoamyl alcohol as described by Gupta et al. (20). The PCR reaction was performed in a final volume of 50 µL. Each reaction contained 1 µL of DNA template, 0.5 µL of each primer, 0.20 mm of each deoxynucleoside triphosphate (dNTPs), 5 µL of 10X PCR buffer, and 1.25 U of Taq polymerase, using 2720 Applied Biosystem thermocycler (life technologies, US). The primer nucleotides was as follows: forward, 5'-TAACAAGGATCCCCTAGTA-3', and reverse 5'-ATTACGC-CAGCATCCTAAG-3'. After initial denaturation at 94°C for 5 minutes, the reaction was followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 45 seconds, with a final extension step of 72°C for 7 minutes.

PCR products were evaluated by 1.5% (w/v) agarose gel electrophoresis in tris-boric acid-EDTA (TBE) buffer, stained with ethidium bromide, and photographed under UV transillumination. The target part of the 26rDNA region was amplified for the *Malassezia* isolates, to produce a PCR product approximately 580bp in length (Figure 1). PCR products were used for further restriction fragment length polymorphism (RFLP) by CfoI (Roche, Germany) enzymes. Digestion was performed by incubating a 20 µL aliquot of PCR products with 10 U of the enzyme in a final reaction volume of 25 µL at 37°C for 3 hours, followed by 2% ethidium bromide agarose gel electrophoresis in a TBE buffer (Figure 2).

4. Results

Direct examination of the specimens was positive in 93.3% (112/120) of questionable PV lesions. However, only 89.3% (100/112) of the positive specimens yielded *Malassezia* in modified Dixon agar. The patients with PV included 60 men and 52 women, aged between 1 and 85 years. Statistically there was no significant difference regarding gender and nor was it observed in the frequency of *Malassezia* species isolated between women and men in each group or between groups ($P = 0.3$). The average age of the patients was 26 years. The prevalence of PV in patients 21-

30 years-of-age was higher than in the other age groups (Table 1). In the present study, no difference was seen in the frequency of *Malassezia* species occurrence depending on the age of the patients. In total, 65% of patients with PV had hyperhidrosis. In 86% of patient samples, yeast and hyphal forms of *Malassezia* were observed in direct microscopy examination of PV lesions.

M. globosa (66%) was the most frequent *Malassezia* species isolated in the PV lesions followed by *M. furfur* (26%), *M. restricta* (3%), *M. sympodialis* (3%), and *M. slooffiae* (2%), respectively. Overall, *M. globosa* and *M. furfur* were the most frequently isolated organisms. The prevalence of *Malassezia* species based on the gender of patients is

shown in Table 2. *Malassezia* species were mainly isolated from the neck and chest. Overall, 36 and 26 isolates were obtained from these sites, respectively. In addition, the lowest number of *Malassezia* species isolated were from the arms and abdomen (Table 3).

A statistical analysis of isolated *Malassezia* species showed that there was no significant relationship between *Malassezia* species and any of the anatomical sites (P values = 0.2). The distribution of sites and *Malassezia* species based on pigmentation, is shown in Table 4. The duration of the disease ranged less than six months in 44% of patients, followed by; six months to one year (26%), one to two years (10%), and over two years (20%).

Table 1. Frequency of *Malassezia* Species According to Age Groups ^a

Age Group	<i>Malassezia</i> Species (n)					Total
	<i>M. globosa</i>	<i>M. furfur</i>	<i>M. restricta</i>	<i>M. sympodialis</i>	<i>M. slooffiae</i>	
1-10 y	6	5	0	0	0	11
11-20 y	15	5	1	1	0	22
21-30 y	25	6	1	1	2	35
31-40 y	11	5	1	0	0	17
41-50 y	5	3	0	1	0	9
> 50 y	4	2	0	0	0	6
Total	66	26	3	3	2	100

^a Values are based on the percentage of *Malassezia* species isolated.

Table 2. *Malassezia* Species Isolated by Gender ^a

Gender	<i>Malassezia</i> Species					Total
	<i>M. globosa</i>	<i>M. furfur</i>	<i>M. restricta</i>	<i>M. sympodialis</i>	<i>M. slooffiae</i>	
Male	39	9	2	2	2	55
Female	27	17	1	1	0	45
Total	66	26	3	3	2	100

^a The values are based on percent of *Malassezia* species isolated.

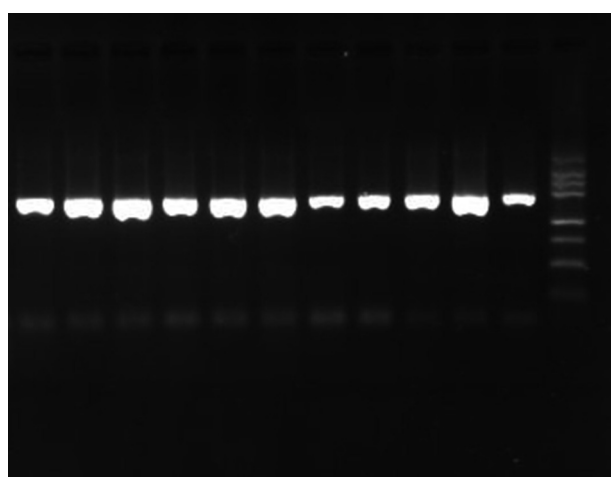
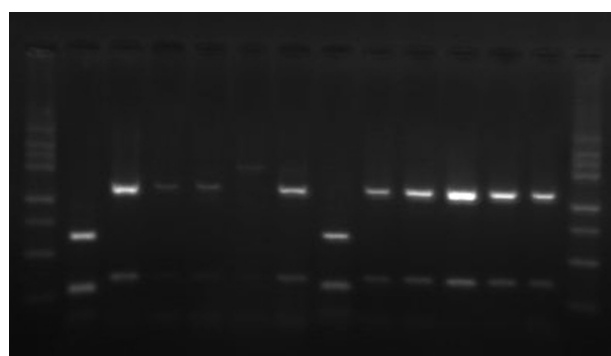
Table 3. Distribution of *Malassezia* Species Isolated According to Clinical Signs ^a

Skin Pigmentation	<i>Malassezia</i> Species					Total
	<i>M. globosa</i>	<i>M. furfur</i>	<i>M. restricta</i>	<i>M. sympodialis</i>	<i>M. slooffiae</i>	
Hyperpigmentation	31	15	2	1	1	50
Hypopigmentation	25	10	1	0	1	37
Both	10	1	0	2	0	13
Total	66	26	3	3	2	100

^a Values are based on the percentage of *Malassezia* species isolated.

Table 4. Distribution of *Malassezia* species Isolated Based on Lesion Area

Species	Anatomical Sites (n)								Total
	Neck	Back	Chest	Face	Arm	Trunk	Limb	Groin	
<i>M. globosa</i>	25	20	17	14	12	10	5	4	107
<i>M. furfur</i>	8	5	6	6	3	4	2	-	34
<i>M. restricta</i>	2	1	1	-	-	-	-	-	4
<i>M. sympodialis</i>	1	1	1	-	-	-	-	-	3
<i>M. slooffiae</i>	-	-	1	-	-	1	-	-	2
Total	36	27	26	20	15	15	7	4	150

Figure 1. Amplicon Size For *Malassezia* Species is 578-584 bp26S rDNA PCR Products before Digestion with CfoI (Lanes 1, 7: *M. furfur*, lanes 2-4, 6, 8-11: *M. globosa*, lane 5: *M. restricta*, lane 14: 100 bp ladder).**Figure 2.** 26S rDNA PCR Products After Digestion With CfoILanes 2, 8: *M. furfur*, with 250, two ~107-113bp (as overlapping) fragments, lanes 3-5, 7, 9-13: *M. globosa*, with 129 and 455bp fragments, lane 6: *M. restricta*, with 581bp fragments, lane 14: 100 bp ladder. *M. furfur* has multiple fragments (59, 30, 21, 2bp) not distinguishable after gel electrophoresis.

5. Discussion

The genus of *Malassezia* consists of at least thirteen different species, which have been identified based on

biochemical and morphological features. However, biochemical and morphological features often do not allow the rapidly and exact characterization of related *Malassezia* species, so molecular approaches need to be used in studies of the epidemiology of *Malassezia*, and pathogenesis and disease caused by the *Malassezia* genus.

The present study used the PCR-RFLP method for the identification of the *Malassezia* genus in order to determine the specific identification of *Malassezia* species and the diagnosis of related infections. *M. globosa* and *M. furfur* were isolated significantly more often than other species in this study; albeit at unequal rates, as *M. globosa* was isolated from 66% of patients with PV. Recent studies from Iran have showed that *M. globosa* is the commonest *Malassezia* species isolated from *Malassezia* skin disorders. In contrast to our results, others studies from Iran have reported lower rates of isolation of *M. globosa* as predominant in different geographical areas at the rate of 41-47.6 (4, 14, 16, 19). In addition, compatible with present studies, *M. globosa* are the major causative agent of PV in other countries (21, 22).

Hedayati et al. (18) reported 58% *M. globosa* from patients with Seborrheic dermatitis. Zomrodian et al. (15), reported that *M. furfur* is the most common *Malassezia* species isolated from psoriasis and healthy individuals. Some investigators from other countries have reported *M. globosa* as the prevailing *Malassezia* species correlated to PV, while others report *M. sympodialis* or *M. furfur* as the species with the highest rates of isolation from PV lesions, however, we found no *M. sympodialis* in our findings. In accordance with our data *M. slooffiae* and *M. restricta* were less commonly isolated species as found in previous studies (23).

On the other hand, similar to other published data, the highest prevalence of PV in our study was observed in the 21-30 year-old group, this is likely to be the result of increased sebum discharge at these ages (4, 24). Previous studies have shown that PV is less common in children (4, 14, 25), but we found 11 cases of PV in children, and this may be due to climate and geographical conditions. Kashan is located on the border of a desert, and as a result it has a hot climate that facilitates the occurrence of *Malassezia* skin infections. Giusiano et al. (25) also reported

9% and 24% of children with *Malassezia* infections in the under 5 and up to 15 years, respectively. Different studies have concluded that PV is rarely found in the aged individual, and we had lower cases of PV in the over 50 year old group.

We found no isolates of *M. obtusa* and *M. pachydermatis* in this study, although in other studies in Iran these species were isolated at a low frequency. In previous reports from Iran, *M. pachydermatis* was not isolated, or it was isolated at a low frequency (8.5%). These differences in the prevalence of *Malassezia* species between our study and other reports may be due to geographical variation and some laboratory techniques such as sampling and diagnostic methods. Racial factors and geographical location may be factors in these differences (11). We have isolated single separated colonies from each lesion site, as suggested by some investigators in patients with PV. However, different studies have isolated more than one species from each sample. Obtaining pure culture from mixed samples is usually very difficult. Due to the fact that, some fast and simple growing *Malassezia* species inhibit other species in the culture.

PV lesions were frequently observed on the trunk and arms (26, 27). The high frequency of PV lesions on the neck in our study is controversial, we also observed a number of patients with multiple lesions (30%). On the other hand, some patients with lesions in unexpected locations, such as the face and limbs, and especially the groin, were diagnosed. The higher ambient temperature of our region probably encourages the spread of PV. Epidemiological studies have mentioned the distribution of some species in defined anatomical sites (27). We observed no differences in the frequency of *Malassezia* species based on the anatomical sites, which might be related to the high number of PV patients. Our findings were compatible to those of other similar studies (17, 25). In this study, the recovery rate of *Malassezia* species from PV lesions was 89.3%. In similar studies, this ratio was different and ranged between 45-90% (4, 17, 18, 22, 27).

In the new taxonomy by Gueho et al. (6), different studies have evolved with the purpose of clarifying the epidemiology and role of the *Malassezia* species in skin disorders. This work will help improve knowledge of *Malassezia* genus epidemiology, especially in the central areas of Iran. Future studies will be necessary in order to explain the ecology and relationships of these species with human disease. Molecular techniques have been useful in the identification and discrimination of *Malassezia* species and this provides information about their epidemiology. By using these methods, the detection and identification of individual *Malassezia* species from clinical samples is made substantially easier.

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Study concept and design: Mehdi Nazeri, Rezvan Talaee, Farzad Katirae. Analysis and interpretation of results: Maryam Ghaderi, Mehdi Nazeri, Mahzad Erami, Azam Kazemi Alavi. Drafting of the manuscript: Mehdi Nazeri and Farzad Katirae. Critical revision of the manuscript: Mehdi Nazeri, Farzad Katirae. statistical analysis: Farzad Katirae

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