

Identification of Different *Malassezia* Species Isolated from Patients with *Malassezia* Infections

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Abstract: Yeasts of the genus *Malassezia* are known as the microflora of human skin and that of many warm-blooded animals; but the different *Malassezia* species can induce superficial skin infections. Best known and most frequent is pityriasis versicolor (PV), a chronic and recurrent skin disease occurring primarily in hot and humid climates. The purpose of the present study was to make use of the metabolic differences and assay techniques, to compare the distribution of *Malassezia* species and gain insight into the epidemiology and ecology of the species identified. In this study, 25 patients with approved (PV) were selected and skin samples were cultured on Sabouraud glucose agar and mDixon. Differentiation of *Malassezia* species was performed using of assimilation of Tweens, catalase reaction, splitting of esculin and growth without addition of lipids. *M. globosa* (42.85%), *M. furfur* (31.4%), *M. sympodialis* (11.42%), *M. pachydermatis* (8.57%) and *M. obtusa* (5.71%) were the most important isolated. Interestingly, in 10 patients two different *malassezia* species were isolated. Regarding to the results of this study, *M. furfur* and *M. globosa* were the most prevalent species in the skin of patients with PV; so these organisms can jointly cause the infections. It is necessary to investigate of epidemiology and ecology of distribution of *Malassezia* species.

Key words: *Malassezia* species • Pityriasis versicolor • Isolation

INTRODUCTION

Yeasts of the genus *Malassezia* are known to be components of the microflora of human skin and that of many warm-blooded animals [1] but are also associated with a variety of disease [2]. Best known and most frequent is pityriasis versicolor (PV) [2], a chronic and recurrent skin disease occurring primarily in hot and humid climates. Although PV had been described at the beginning of nineteenth century [3], until recently classification of its etiologic agent was a matter of debt. This controversy may be caused by various morphological features and fastidious growth requirements of *Malassezia* yeasts in vitro. The genus of *Malassezia* has undergone several taxonomic revisions [3-5]. In the last reclassification by Gueho *et al.*, seven distinct species were recognized with in this genus, namely *M. furfur*, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae* [3].

There is only scanty information about the epidemiology and ecology of *Malassezia* species available and the clinical significance of this species is not completely recognized [3]. Further differentiating systems, based on biochemical differences and suitable for routine diagnosis, were published recently [1]. Sugita *et al.* [2005] reported that *M. globosa* and *M. restricta* were the most frequently isolated species from patients with pityriasis versicolor [19]. The purpose of the present study was to make use of these metabolic differences and assay techniques, to validate the methods and possibly gain insight into the epidemiology and ecology of the species identified.

PATIENTS AND METHODS

Isolates: 35 *Malassezia* species, from 25 patients referred to the Mycology Research Center, University of Tehran, Iran were selected to identify the exact confirmation.

Culture: The isolates were cultured on Sabouraud glucose agar and mDixon agar, containing 0.05% chloramphenicol (Merck, Darmstadt, Germany) and 0.05% cycloheximide (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% cycloheximide devoid of lipids. Incubation was performed at 31°C for one week.

Tween Assimilation Test: According to the method reported by Guillot *et al.* [3, 8], ability to utilize different Tween compounds as a unique lipid supplement by *Malassezia* species was evaluated. Briefly, yeast suspension (2×10^5 to 3×10^5 cfu/ml) was made in 1 ml sterilized distilled water and poured into plate containing SGA with 0.05% chloramphenicol and 0.05% cycloheximide 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 [8].

Assimilation of Cremophor EL: Assimilation of Cremophor EL (PEG-(35)-Caster oil; Sigma, St. Louis, MO, USA) was examined by means of the agar diffusion test as follows [1,9]: Briefly, 10 ml sterile selective agar for pathogenic fungi (Merck, Darmstadt, Germany) was melted and allowed to cool to about 50°C. Yeast suspension (2×10^5 to 3×10^5 cfu/ml) was made in 1 ml sterilized distilled water and poured into plate containing the medium. The inoculum was then spread evenly. Once the medium had solidified, one hole was made by means of a sterile 6-mm punch and filled with 50 µl of Cremophor EL.

The plates were incubated at 31°C for 10 days and assessed for growth around the individual wells after 2,4,6,8 and 10 days.

Splitting of Esculin: To improve the differential diagnosis of different species of *Malassezia* as published previously [1, 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 esculetin 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* [1, 6]. *M. furfur* causes weak staining. *M. pachydermatis* has a variable reaction [6]. 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 [6,10].

Pigment Induction Medium (P-Agar): The medium for induction of pigment synthesis was used as follows [2, 11]: 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.

RESULTS

From 25 patients with *Malassezia* infection, 60% of the cases were male. The range and median age of patients were 13-55 and 35 years, respectively. The highest prevalence of *Malassezia* infection was seen in patients with 30-55 years of age.

Table 2 shows the results of the specimens concerning assimilation of Tween, assimilation of Cremophor EL, splitting of esculin, catalase reaction, pigment synthesis, growth at 40°C and growth without addition of lipids. The key characteristics of the species of *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*

Table1: Scheme for the identification of *Malassezia* isolates (6).

Growth With

Strains	No lipid supplement	High concentration of Tween 20	Tween 40	Low concentration of Tween 80	Cremophor EL	Pigment synthesis	Splitting of esculin	Catalase reaction	Growth 40°C
<i>M. pachydermatis</i>	+	+	+	+	V	(+)	V	V	+
<i>M. furfur</i>	-	+	+	+	V	+	-	+	+
<i>M. sympodialis</i>	-	-	+	+	-	-	+	+	+
<i>M. globosa</i>	-	-	-	-	-	-	-	+	-
<i>M. obtusa</i>	-	-	-	-	-	-	+	+	-

-1=*M. globosa*; 2=*M. furfur*; 3=*M. sympodialis*; 4=*M. pachydermatis*; 5=*M. obtusa*

-+: Positive; -: Negative; V: Variable reaction; (+): Delayed positive

Table 2: Frequency of *Malassezia* species according to different standard tests

<i>Malassezia</i> isolates	Solely isolates Number (%)	Mixed isolates Number (%)	Total isolates Number (%)
<i>M. globosa</i>	8 (22.85)	7 (20)	15 (42.85)
<i>M. furfur</i>	2 (5.71)	9 (25.71)	11 (31.42)
<i>M. sympodialis</i>	3 (8.57)	1 (2.86)	4 (11.42)
<i>M. pachydermatis</i>	2 (5.71)	1 (2.86)	3 (9.57)
<i>M. obtusa</i>	0 (0)	2 (5.71)	2 (5.71)
Total	15 (42.85)	20 (57.14)	35 (100)

Table 3: Frequency of *Malassezia* isolates based on age, sex and anatomical site.

Age	Location																				Total
	Ear		Chest		Neck		Abdomen		Axilla Head		Back		Groin		Planta Body and Groin		Back and Head		Abdomen		
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	
<19								4
20-30			9
431-39			8
940<	14
Total	1	1	4	4	0	1	0	2	1	1	2	2	1	1	0	2	2	3	1	2	35

-F: Female; M: Male

and *M. pachydermatis* has shown on Table 1 [6]. Ten of these consist of mixed cultures. Using the combination identification system, the most commonly isolated species was *M. globosa* (42.85%), followed by *M. furfur* (31.4%), *M. sympodialis* (11.42%), *M. pachydermatis* (8.57%) and *M. obtusa* (5.71%). Of the *Malassezia* isolates under study, 11(44%) growth with Cremophor EL, 6(24%) splitting of esculin, 7(28%) pigment synthesis, 6(24%) growth at 40°C, which is considered to be indicated *M. furfur*, *M. sympodialis* and *M. obtusa*. For identification and differentiation of *M. globosa* and confirmation of the other species of *Malassezia* was accomplished assimilation of Tweens (Table 2).

DISCUSSION

Recently, different *Malassezia* species have been considered as etiologic agents of PV, folliculitis, seborrheic and atopic dermatitis, dandruff, onychomycosis and systemic infections. Although PV has worldwide occurrence, its frequency is variable and depends on different climatic, occupational and socio-

economic conditions [3, 12, 13]. This disease is prevalent in Iran, in which almost 6% of all dermatosis and approximately 30% of dermatomycoses are due to these lipophilic yeasts [3, 14]. The present study aimed to identification of *Malassezia* species from patients with *Malassezia* infections.

The highest prevalence of PV in present study was observed in 30-55 year-old group (table 3); while in other investigation [3, 15, 16] this range was 20-30.

The role of sex in propensity to development of PV is still unclear [3].some studies found that PV is more common in men than women, just as in this survey 60% of the cases were male (table 3), while others indicated that the incidence of this infection is higher in women [3, 17], which may be due to extra attention of women to beauty and skin hygiene. However, similar to many reports [3, 15, 16, 18], we found no differences in development of PV among both sexes.

Although the morphology characteristics (colony and microscopic examination) for *Malassezia* yeast is used for primary identification; but they do not provide sufficient information for specific identification of isolates.

For this reason, In order to avoid any confusion, we carried out physiological tests. There are two simple preliminary tests, namely the subculture on SGA (at 31°C) and the catalase reaction. If the growth on SGA is observed, the organism is the non lipid-dependent species *M. pachydermatis*, which confirmed in 2 isolates from patients with otomycosis.

M. pachydermatis infection in human is rare. These patients are the staff of Faculty of Veterinary Medicine and it is speculated that the yeast was transmitted from infected pet animal.

All lipid-dependent species, except *M. restricta*, exhibit a positive catalase reaction. It is important that by using of PCR methods for *M. globosa* and *M. restricta* are the most frequently isolated species from patients with PV. These cultured-based methods are based by different growth rates and culture requirement for different species [8, 19] and *M. restricta* may not be detected in samples in which a catalase reaction-positive *Malassezia* species is mixed. Based on many studies, more than one species can be recovered from each sample [3, 20]. Interestingly, in 11 samples, different *Malassezia* species were jointly isolated. So we carried out the collections of different tests and for exact identification of *M. restricta*, molecular technique should be used and we could not isolated this species.

In the present study, *M. furfur*, which is thought to cause PV by pigment production [1, 2], was confirmed by both cremophor EL assimilation [1, 9] and the ability to synthesize pigment (Table 1).

A new minimal medium, consisting of the amino acid tryptophan and a lipid source, allowed growth only of the species *M. furfur* and induced the formation of a brown pigment that diffused into the agar. On the minimal medium the effect was highly specific for *M. furfur*. Both *M. sympodialis* and *M. pachydermatis* strains as well as the other yeast species tested failed to grow and produce pigment on this medium. By contrast, Mayser *et al.* [11] showed that a few of *M. pachydermatis* strains produced pigment formation on the P-medium. Also, they investigated that in contrast to *M. furfur* pigment formation occurred after a markedly longer incubation time (4 weeks unlike 3-5 days). In accordance with Mayser *et al.* [11], the *M. pachydermatis* isolates showed no pigment formation after 4 weeks and positive results were obtained for *M. furfur* isolates during 5-7 days.

Cremophor EL is the one lipid source as well as Tweens that provides the growth of *M. furfur*. Despite of the above mentioned, Midgley *et al.* (6, table 2) explained a variable reaction for *M. furfur* and *M. pachydermatis*.

Our results are accordance with other investigations, as *M. pachydermatis* isolates were negative.

Using of esculin agar, another test for identification of *M. sympodialis* and *M. obtusa*, significant brown staining of more than a third of the medium was observed to these species that showed positive reaction (table 2).

The Tween test allows the differentiation of different *Malassezia* species. The precipitate provides typical features combined or not with growth. This phenomenon may correspond to the hydrolysis of Tweens with precipitation of the corresponding insoluble fatty acid. However, when the Tweens allow a growth, the precipitate is absent or remains weak likely because of the acidification of the medium.

Since *M. globosa* and *M. obtusa* have Tween assimilation patterns, esculin agar test was used.

Sometimes, it may not interpret the Tween assimilation accurately, so for differentiation of *M. sympodialis* and *M. obtusa*, have a positive reaction with splitting of esculin test, the growth at 40°C accomplished (table 2).

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