Biochemical Characterization and Genotyping by RAPD-PCR Analyses of *Malassezia* spp. From Pityriasis Versicolor and Seborrhoeic Dermatitis Patients

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Abstract: This work was carried out to investigate the biochemical-physiological characteristics and genetic variations of *Malassezia* species isolated from pityriasis versicolor and seborrhoeic dermatitis patients. Twenty two pityriasis versicolor and seborrhoeic dermatitis outpatients at Razi Hospital of Tehran University were included in this study. The collected samples from patients were cultured and identified for *Malassezia* spp. by the conventional techniques based on the morphological, biochemical and physiological characteristics. Random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR) was applied for genetic variations of the isolates. Results revealed that *M. furfur* was the prevalent species isolated from pityriasis versicolor either alone (40 %) or in combination with *M. globosa* (40 %); while *M. globosa* was the most frequently isolate from seborrhoeic dermatitis either alone (41/7 %) or combination with *M. furfur* (8/3 %). The RAPD analysis among *Malassezia* isolates was counteracted from 238 amplified products in 81 separable positions and indicated that all *Malassezia* isolates were composed of nine genetically distant groups. In conclusion, we found different physiological characteristics and genetic variations of *Malassezia* isolates from all patients. Moreover, RAPD-PCR can be used as a powerful tool in epidemiological investigation of dermatological disorders associated with *Malassezia* species such as pityriasis versicolor and seborrhoeic dermatitis.

Key words: Pityriasis versicolor · Seborrhoeic dermatitis · Malassezia species · RAPD-PCR

INTRODUCTION

Members of the genus *Malassezia*, lipophilic yeasts, are commonly isolated from human skin flora and are mostly found in sebum areas of the skin such as the trunk, back, scalp, face, neck and shoulders. Currently, 14 species have been identified within this genus, *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. slooffiae*, *M. restricta*, *M. pachydermatis*, *M. nana*, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. caprae*, *M. equine* and *M. cuniculi* which are associated with several common dermatological disorders such as pityriasis versicolor (PV), seborrhoeic dermatitis (SD), atopic dermatitis and folliculitis [1-11].

Only few culture studies have been made using the new classification of the *Malassezia* yeasts [12]. However, it is important to look at the distribution of the various *Malassezia* species not only on normal skin but also on the skin of patients with *Malassezia*-associated skin diseases. PV presents as scaly hypo- or hyper pigmented lesions usually on the trunk and back of affected individuals [13], although in extensive disease, lesions may occur on almost any body site. There is a postulated that this disease occurs when *Malasseiza* yeasts that normally colonize the skin change from the round yeast form to a pathological mycelia form, which then invades the stratum corneum of the skin [14].

The most common disease associate with *Malassezia* yeasts is SD (and the related condition, dandruff), an inflammatory condition occurring on the scalp, face and trunk in 1-3% of the general population. There is a higher incidence of these dermatoses in immune-compromisd patients, especially those with AIDS, ranging from 34% to 83% [15].

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Published epidemiological data suggest geographical variations in the rate of the isolated *Malassezia* species. *Malassezia* species can be identified through morphological, biochemical and molecular characteristics. The identification system based on different biochemical physiological methods may fail to identify atypical isolates or new species [16] and molecular typing methods have been developed to evaluate the distribution of different *Malassezia* subtypes within a given disease spectrum [17]. Several techniques have been used to acquire epidemiological information of dermatological disorders caused by *Malassezia* yeast. These include karyotyping by pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP), polymerase chain reaction followed restriction enzyme analysis (PCR-REA) and nested PCR [9, 14, 18- 22].

The intra-species subtypes obtained with RAPD from samples of *Malassezia* species suggest the presence of genetic population differences that may be an important tool for epidemiological investigation [18, 23].

The aim of the present study was to investigate the biochemical-physiological characteristics and analyze the DNA profile by RAPD-PCR of *Malassezia* species isolated from PV and SD patients.

MATERIALS AND METHODS

Twenty two PV and SD outpatients at Razi Hospital of Tehran University were included in this study. The subjects were 10 patients with PV and 12 patients with SD. Samples were collected by the swab method from the sites mentioned in table 1 and cultured on modified Dixon agar and Sabouraud dextrose agar (SDA), containing 0.05% chloramphenicol (Merck, Darmstadt, Germany) and 0.05% cycloheximide (Sigma, St Louis, MO, USA). All the plates were incubated at 31°C for 10 days and were monitored daily. Suspected colonies of *Malassezia* spp. were identified according to Gueho *et al.* [24] and Mayser *et al.* [25].

Table 1: Identification of Malassezia species based on physiological tests in patients studied

Isolate	Skin disease / Source	Growth without lipid	Catalase reaction	Cremophor EL	Esculin	Pigment synthesis	Growth 40°C	Tween 20	40	60	80	Malassezia spp.
m1	PV/ Trunk	-	+	+	(+)	+	+	+	+	+	+	M. furfur
m2	SD/Ear	+	+	-	-	-	+	+	+	+	+	M. pachydermatis
m3	SD/Neck	-	+	-	-	-	-	-	-	-	-	M .globosa
m4	PV/Trunk	-	+	+	-	+	-	-	-	-	-	M. globosa + M. furfur
m5	PV/Abdomen	-	+	-	-	-	-	-	-	-	-	M. globosa
m6	PV/Axilla	-	+	+	-	+	+	+	+	+	+	M. furfur
m7	SD& Dandruff /Groin & Scalp	-	+	+	-	+	+	+	+	+	+	M. furfur
m8	PV/Back	-	+	+	-	+	+	+	+	+	+	M. furfur
m9	SD/Groin	-	+	-	-	-	-	-	-	-	-	M. globosa
m10	PV/Trunk	-	-	+	-	+	+	+	+	+	+	M. furfur
m11	PV/Back	-	+	+	-	-	-	-	-	-	-	M. globosa + M. furfur
m12	SD/Abdomen	-	+	+	-	+	-	-	-	-	-	M. globosa + M. furfur
m13	SD/Planta	-	+	-	+	-	+	-	+	+	+	M. sympodialis
m14	PV/Trunk	-	+	+	-	+	-	-	-	-	-	M. globosa + M. furfur
m15	SD/Trunk	-	+	-	-	-	-	-	-	-	-	M. globosa
m16	Dandruff/Scalp	-	+	-	+	-	+	-	+	+	+	M. sympodialis
m17	SD/planta	-	+	-	+	-	+	-	+	+	+	M. sympodialis
m18	Dandruff/Scalp	-	+	-	-	-	-	-	-	-	-	M. globosa
m19	SD/trunk	-	+	+	+	-	+	+	+	+	+	M. furfur+M. sympodialis
m20	PV/Back &Abdomen	-	+	+	-	+	-	-	-	-	-	M. globosa + M. furfur
m21	Dandruff/Scalp	-	+	-	-	-	-	-	-	-	-	M. globosa
m22	PV/Back	-	+	-	-	-	-	-	-	-	-	M. globosa

+: positive, -: negative, (+): weak positive

Table 2: Sequences of primers and amplification program for RAPD-PCR

Target	Sequence(5' to 3')	Amplification program						
		1	2	3	4	5	6	
FM1	AGCCGCCTCCATGGCCCCAGG	95°C 1min	94°C 1min	40°C 2min	72°C 1min	72°C 5min	4°C	2~4 40 cycle
M13	GAGGGTGGCGGTTCT	95°C 6min	94°C 45sec	32°C 90sec	72°C 90sec	72°C 8min	4°C	2~4 40 cycle
B2	ATGGATCGG	95°C 5min	94°C 45sec	36°C 45sec	72°C 90sec	72°C 10min	4°C	2~4 40 cycle
P10	GACAGACGCG	95°C 1min	95°C 15sec	39/7°C 45sec	72°C 90sec	72°C 5min	4°C	2~4 30 cycle
A4	AATCGGGCTG	95°C 5min	94°C 45sec	36°C 45sec	72°C 90sec	72°C 10min	4°C	2~4 40 cycle
A10	GTGATCGCAG	95°C 5min	94°C 45sec	40°C 45sec	72°C 90sec	72°C 10min	4°C	2~4 40 cycle

Following species identification, the isolated strains were seeded on solid Dixon medium and incubated for 5 days at 32°C to obtain microorganisms at the exponential growth phase. DNA was extracted as described by Liu *et al.* [26]. DNA concentration in the each sample was measured by use of spectrophotometer at λ=260 nm. To confirm the quality of the nucleic acid extracted, DNA was resolved by electrophoresis on a 1% (w/v) agarose gel. Total DNA bands were visualized after stain with ethidium bromide using molecular Analyst™ software (1.4.1. version, Bio-Rad) in the GEL DOC 1000 equipment. The DNA extracts were stored at -20°C.

The following primers were used: B_2, FM_1, M_{13}, P_{10}, A_4 and A_{10} for RAPD reaction. The PCR was carried out in 25 µl volumes containing 100 ng genomic DNA (approximately 1.2 µl), 12.5 µl master mix [(10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM of each dNTPs and 2.5 units of Taq DNA polymerase) (Sinagen Company)], 0.4 µM primer (approximately 1.3 µl) (Sinagen Company) and 10 µl distilled water. RAPD-PCR was performed in a Biotech Thermocycler system programmed for optimal amplification conditions for every primer. The details of the sequences of the primers and amplification programs for RAPD-PCR are listed in table 2.

RAPD products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel and 100bp ladder DNA (Fermentans) was used as molecular weight marker. Gels were stained with ethidium bromide, visualized by UV illumination and photographed with a Polaroid camera.

For data analysis, The RAPD-PCR was performed at least twice. The presence or absence of a marker was scored as 1 or 0, respectively. Similarity coefficient between two isolates were calculated according to the formula of Nei and Li [27] as $F = 2N_{xy}/N_x + N_y$, where N_{xy} was the number of common fragments between two isolates and N_x and N_y were the number of fragments in isolates X and Y, respectively. The genetic distance was evaluated through Euclidean distance. The dendrogram was constructed based on the unweighted pair-group method using arithmetic averages (UPGMA) [28]. Pooled data from six primers were used for this calculation.

RESULTS

Table 1 shows the results of morphological and biochemical-physiological characteristics of twenty two strains of *Malassezia* isolated from PV and SD patients.

The primers P_{10}, FM_1 and M_{13} produced more polymorphic and reproductive profiles. Pooled data from six primers gave a total of 238 clearly amplified PCR bands in 81 different positions. The size of the fragments ranged from 0.47 to 52 Kb and 77.77% bands were larger than 1 Kb (data not shown).

All PCR fragments obtained were used for genetic distance analysis. The dendrogram constructed from the pair wise similarity among all *Malassezia* isolates demonstrating that the tested isolates of *Malassezia* were grouped into 9 distinct groups (Figure1). Within each group, members share more than 80% similarity of the RAPD bands pattern; i.e. the average similarity of group 1 was more than 90%. Furthermore, within the groups, a cluster of 99% identity was found: i.e. cluster 1; m_3 and m_{11}. Group 1, the largest group, contained 13 of 22 isolates. The next large group was7, containing 2 of 22 isolates. The other groups were minor, containing 1 *Malassezia* isolate per group.
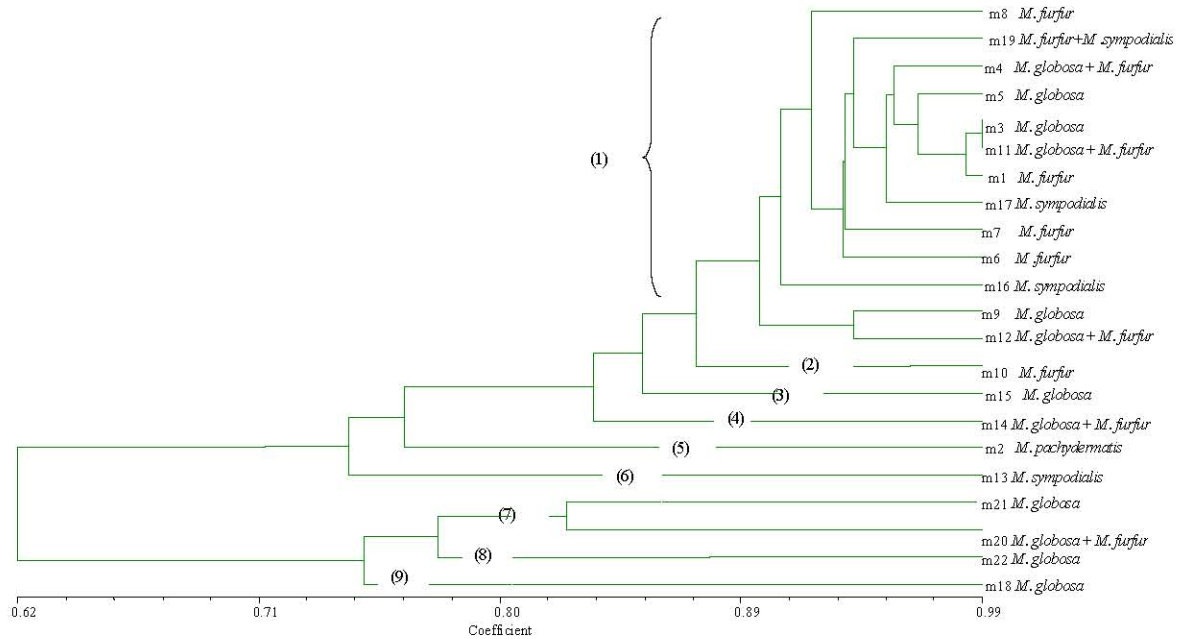
Fig. 1: Dendrogram constructed by UPGMA method of different Malassezia species derived from RAPD assays generated by using six primers.

DISCUSSION

The expansion of the genus Malassezia has generated interest in the epidemiological investigation of the distribution of new species in a range of dermatomycoses. Classical identification of Malassezia species is based on morphological, biochemical and physiological characteristics. By using the combination of different biochemical and physiological methods, it was reported that Malassezia species in mixed cultures are frequently found both in animals and in humans [29, 30]. Four Malassezia species were recovered from PV and SD patients. M. furfur was the prevalent species isolated from PV either alone (40 %) or in combination with M. globosa (40 %); while M. globosa was most frequently isolated from SD either alone (41/7 %) or combination with M. furfur (8/3 %). M. pachydermatis, known as an agent of external otitis in cats and dogs, has also been introduced into the intensive care nursery on the hands of the health care workers and hand colonization may have perpetuated the transmission of the organism from patient to patient (i.e. premature neonates) within the nursery [31, 32]. In our work, this Malassezia species (8/3%) was isolated from the staff of Faculty of Veterinary Medicine with SD and it is speculated that the yeast was transmitted from infected pet animal.

The occurrence of mixed cultures of different Malassezia species was reported by Salah *et al.* [33], Gaitanis *et al.* [17], Zomorodian *et al.* [34], Moniri *et al.* [35] and Khosravi *et al.* [36] that are similar to the present work.

Similar to our findings, M. furfur was predominantly detected from the lesions of PV in Brazil [15] and Indonesia [37]. Also, Makimura *et al.* [38] in Japan noted that M. furfur and M. sympodialis were recovered from PV cases. Zomorodain *et al.* [34] in Iran reported that M. furfur was identified as the most isolated species in hospitalized neonates.

In contrast to these studies, M. globosa is the causative agent of PV in Japan [21, 39], Greece [17], Spain [40, 41], India [42], Tunisia [33], Turkey [43], Bosnia and Herzegovina [44] and Iran [45, 46], while the trend is reversed in Canada with M. sympodialis being the commonest PV isolated [47].

Thomas and Dawson in USA [48] showed that M. globosa and M. restricta predominated on dandruff scalp. Similar to the present study, Gupta in Canada [49] reported that M. globosa was most frequently isolated from SD patients. Likewise, Ho Oh *et al.* in Korea [50] showed that M. restricta was dominant in patients with SD; while M. obtuse in Sweden [12] and M. furfur in Brazil [15] and Iran [51] were cultured from SD lesions at higher rates. These observations suggest

that the geographical variations in the distribution of Malassezia species in PV and SD lesions may reflect climatic differences.

In this work, the assessment of randomly amplified polymorphic DNA was established as a molecular epidemiological tool. Major advantages of the RAPD-PCR are flexibility, technical simplicity and high discriminatory power. Low levels of misclassification and high levels of specificity make RAPD-PCR an efficient, sensitive and suitable mean of distinguishing closely related strains. The importance of RAPD typing method have been demonstrated in several studies of Malassezia genera and other fungi that are poorly characterized allowing a rapid evaluation of genetic diversity of these species [15].

Phenogram of Malassezia species based on UPGMA method derived from RAPD assays and generated by using six primers showed that M. globosa isolated from PV and SD patients is clustered to five (clusters 1, 3, 7, 8 and 9) and three (clusters 1, 4 and 7) alone or in combination with M. furfur, respectively. M. furfur isolated from all patients is clustered to two (clusters 1 and 2) and three (clusters 1, 4 and 7) alone or in combination with M. globosa or M. sympodialis, respectively. In addition, M. sympodialis isolated from the studied patients was two (groups 1 and 6) and one (group 1) alone or in combination with M. furfur, respectively. M. pachydermatis isolated from the staff of Faculty of Veterinary Medicine with SD lesions was clustered to one (cluster 5).

In conclusion, the data presented in this study indicate that specific genotypes are involved in skin diseases caused by Malassezia species such as PV and SD. Moreover, RAPD-PCR can be used as a powerful tool in epidemiological investigation of dermatological disorders associated with Malassezia species.

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