Biochemical Characterization and Genotyping by RAPD-PCR Analyses of Malassezia spp. From Pityriasis Versicolor and Seborrheic 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 seborrheic dermatitis patients. Twenty-two pityriasis versicolor and seborrheic 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 seborrheic 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 seborrheic dermatitis.

Key words: Pityriasis versicolor · Seborrheic 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), seborrheic 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 hyperpigmented 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 Malassezia 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 associated 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-compromised 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>
<thead>
<tr>
<th>Isolate</th>
<th>Skin disease / Source</th>
<th>Growth without Pigment synthesis</th>
<th>Growth at 40°C</th>
<th>Pigment growth at 20°C</th>
<th>Pigment growth at 40°C</th>
<th>Pigment growth at 60°C</th>
<th>Pigment growth at 80°C</th>
<th>Malassezia spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1 PV/Trunk</td>
<td>- + + (+)</td>
<td>+ + + + + + +</td>
<td>+ + + + + + +</td>
<td>M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m2 SD/Ear</td>
<td>+ + + -</td>
<td>- + + + + +</td>
<td>- + + + + +</td>
<td>M. pachydermatis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m3 SD/Neck</td>
<td>- + - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m4 PV/Trunk</td>
<td>- + - +</td>
<td>+ - - - -</td>
<td>+ - - - -</td>
<td>M. globosa + M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m5 PV/Abdomen</td>
<td>- + - -</td>
<td>- - - -</td>
<td>- - - -</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m6 PV/Asilla</td>
<td>- + - +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>M. furfur</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>m7 SD &amp; Dandruff</td>
<td>/Groin &amp; Scalp</td>
<td>- + + +</td>
<td>- + + + +</td>
<td>M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m8 PV/Back</td>
<td>- + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m9 SD/Groin</td>
<td>- + - -</td>
<td>- - + +</td>
<td>- - + +</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m10 PV/Trunk</td>
<td>- + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m11 PV/Back</td>
<td>- + - +</td>
<td>- - - +</td>
<td>- - - +</td>
<td>M. globosa + M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m12 SD/Abdomen</td>
<td>- + + -</td>
<td>- + - +</td>
<td>- + - +</td>
<td>M. globosa + M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m13 SD/Planta</td>
<td>- + - +</td>
<td>- + - +</td>
<td>- + - +</td>
<td>M. sympodialis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m14 PV/Trunk</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. globosa + M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m15 SD/Trunk</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m16 Dandruff/Scalp</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. sympodialis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m17 Dandruff/Scalp</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. sympodialis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m18 Dandruff/Scalp</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m19 SD/Trunk</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. sympodialis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m20 PV/Back &amp; Abdomen</td>
<td>- + + +</td>
<td>+ - -</td>
<td>- + -</td>
<td>M. globosa + M. furfur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m21 Dandruff/Scalp</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m22 PV/Back</td>
<td>- + - +</td>
<td>- + -</td>
<td>- + -</td>
<td>M. globosa</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

+: positive, -: negative, (+): weak positive
Table 2: Sequences of primers and amplification program for RAPD-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence(5’ to 3’)</th>
<th>Amplification program</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>FM1</td>
<td>AGCCGCCTCCATGGCCCAAGG</td>
<td>95°C 1min</td>
</tr>
<tr>
<td>M13</td>
<td>GAGGGTCGCGGTTCTCT</td>
<td>95°C 6min</td>
</tr>
<tr>
<td>B2</td>
<td>ATGGATCGGG</td>
<td>95°C 5min</td>
</tr>
<tr>
<td>P10</td>
<td>GACAGACCGGG</td>
<td>95°C 1min</td>
</tr>
<tr>
<td>A4</td>
<td>AATCGGGCCTG</td>
<td>95°C 5min</td>
</tr>
<tr>
<td>A10</td>
<td>GTGATGCCAG</td>
<td>95°C 5min</td>
</tr>
</tbody>
</table>

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 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 staining 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: B2, FM1, M13, P10, A4, and A10 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 (Fermentas) 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 = 2Nxy/Nx + Ny, where Nxy was the number of common fragments between two isolates and Nx and Ny 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 P10, FM1 and M13 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, and m11. Group 1, the largest group, contained 13 of 22 isolates. The next large group was?, 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 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. Zomorodian 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. obtusa 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 (clusters 1 and 6) and one (cluster 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.

REFERENCES


