Distribution of *Malassezia* species in Mexican seborrheic dermatitis patients

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Yeast of the *Malassezia* genus are linked to seborrheic dermatitis (SD) in humans; however, etiological species causing this disease can vary according to their geographical location. *M. globosa* and *M. restricta* are the most often isolated microorganisms and can be found in the skin of patients with SD. Nevertheless, species identification by molecular methods and the relationship among etiological agents and the clinical severity of the disease have not been determined in Mexican patients. The goal of this study was to analyze the prevalence of *Malassezia* species in the skin of SD patients by molecular methods in order to establish their distribution according to the severity of the disease in Monterrey, Nuevo Leon, Mexico. Skin samples from patients with SD (n = 60) were obtained by scraping and were cultured on modified Dixon agar. The *Malassezia* colonies were identified by amplification of the D1/D2 regions of 26S rDNA by polymerase chain reaction (PCR) and subsequent sequencing and BLAST analysis in GenBank. The positive *Malassezia* culture rate was 48.3%. The most commonly isolated species were *Malassezia furfur* (20%), *Malassezia globosa* (16.7%), *Malassezia sympodialis* (6.7%), *Malassezia restricta* (3.3%) and *Malassezia slooffiae* (1.7%). No significant difference was found in the distribution of *Malassezia* species according to disease severity.

**Key words:** Seborrheic dermatitis, *Malassezia*, LSU rDNA D1/D2, yeasts, *Malassezia furfur*, *M. globosa*.

**INTRODUCTION**

*Malassezia* species are lipophilic yeasts that are part of the flora of human skin and skin of warm blooded animals (Crespo et al., 1999; Matousek and Campbell, 2002; Scott et al., 2001). These yeast have been associated
with diseases, such as seborrheic dermatitis, atopic dermatitis (Hirai et al., 2004; Sugita et al., 2002, 2004), folliculitis (Cabello and Cervero-Vivas, 2004), catheter-associated neonatal sepsis (Falk et al., 2005; Van Belkum et al., 1994) pityriasis versicolor (Erchiga et al., 2000; Gaitanis et al., 2006) and external otitis (Cafarchia and Otranto, 2004; Crespo et al., 2000). Malassezia genus taxonomy has been recently restructured based on molecular DNA analysis and lipid requirements; Malassezia genus has been divided into seven species, including Malassezia furfur (Robin) [Baillon, 1889], Malassezia pachydermatis (Weidman) [Dodge, 1935], Malassezia sympodialis [Simmons and Gueho, 1990], Malassezia globosa Midgley (Guého et al., 1996), Malassezia obtusa, Malassezia restricta and Malassezia slooffiae. M. pachydermatis is the only non-lipid-dependent species. Currently, the genus Malassezia is composed by 14 species just accepted: Malassezia dermatis, Malassezia japonica, Malassezia yamatoensis, Malassezia nana, Malassezia caprae, Malassezia equine and Malassezia cuniculi. SD is an inflammatory dermatosis characterized by chronic eczema with erythematous plaques and dry or greasy scales (Gerd and Thomas, 2008). Usually this disease occurs in young adults in areas rich in sebaceous glands such as the face, scalp and upper trunk (Del Rosso, 2011; Tajima et al., 2008). SD is more frequent in patients with HIV than in the general population with a prevalence of 30-55% (Tajima et al., 2008; Gandra et al., 2006; Gupta and Bluhm, 2004; Rincón et al., 2005; Schechtman et al., 1995) as compared to 1-3% of the general population, being more prevalent in men than in women (Johnson and Roberts, 1977; Naldi and Rebora, 2009). Recently, published studies have demonstrated that Malassezia spp. may be a normal host in human skin (Fredericks, 2001; Mastrolonardo et al., 2003; Pierard, 2003; Schwartz et al., 2006). In contrast, the therapeutic response of SD to antifungal treatment suggests the role of the yeast as an etiological factor (Sugita et al., 2002). Some studies have investigated the relationship between SD and the presence of Malassezia yeast in human scales and observed that M. restricta and M. globosa are the most commonly associated species (Devlin, 2006; Zarei-Mahmoudabadi et al., 2013; Zhang et al., 2013). The association between clinical severity and each species remains unclear (Picardo and Camelli, 2008).

In the present study, the prevalence of Malassezia spp. in patients with SD lesions on the face and scalp was analyzed by molecular techniques and correlated the etiologic agents with clinical severity.

MATERIALS AND METHODS

Study subjects

Sixty patients with SD with active facial and scalp involvement (32 women and 28 men, with a mean age of 37 years) were selected. In each case, the extent and severity of the lesions were assessed.

Only patients who had not used any topical or oral treatment during the previous month were included. A survey to assess disease onset, first degree relatives affected, stress-related outbreaks and history of other dermatological diseases was performed. Informed consent from all participants was obtained according to the requirements of the institutional ethics committee. Sampling was performed from the facial and scalp lesions, and the skin scales removed with a sterile blade transferred to the laboratory in a sterile Petri dish, were processed at the microbiology laboratory of the Faculty of Veterinary Medicine. The samples were cultured on modified Dixon’s medium containing chloramphenicol (0.5%) and cycloheximide (0.5%). Plates were incubated at 32°C for 7-14 days, and identification of Malassezia species was performed according to the methods described by Guého et al. (1996) and Guillot et al. (1996). The presence of the yeast Malassezia was microscopically observed using Gram stain from the colonies grown on modified Dixon’s medium, and the results were confirmed by amplified PCR of the variable D1 and D2 regions of the 26S rRNA gene, using the conserved fungal oligonucleotide primers NL1 and NL4 (O’Donnell, 1993).

Ethical approval

All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Samples

DNA extraction

For DNA isolation, cells were harvested from 4-5 day old cultures in modified Dixon’s medium, and the DNA was prepared as described previously by Ferrer et al. (2001). Briefly, the yeasts cells were incubated for 1 h at 65°C in 500 μL extraction buffer (Tris-HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted using phenol : chloroform : isoamyl alcohol (25:24:1). Then, 65 μL of 3 M sodium acetate and 75 μL of 1 M NaCl were added to 350 μL of the supernatant, and the resulting volume was incubated at 4°C for 30 min. The DNA was recovered by isopropanol precipitation; washed with 70% (v/v) ethanol; dried under a vacuum; and resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). The Ultraspec 3000 pro (Amersham Bioscience, GE Healthcare, DF, México) was used to determine the DNA concentration.

PCR and sequencing of isolates

Genomic DNA (100 ng) obtained from samples that were suspected to have Malassezia spp. was used as a template in a PCR reaction with a final concentration of 10 mM buffer 10X (BioTherm), 0.1 mM dNTP’s, 1.5 mM MgCl, 0.198 μM each primer (NL1 5’ GCA ATC AAT AGC GGG AGG AAG A-3; NL4 5’ GTT CC CGT TTT CT A AAG C G GG-3) (O’Donnell, 1993) and 1 U Taq DNA polymerase (Bio Therm Piscataway, NJ 08854 USA). PCR conditions consisted of a pre-denaturing step at 94°C for 5 min and 30 amplification cycles: denaturation at 94°C for 45 s; annealing for 1 min at 51°C; extension at 72°C for 3 min; and a final extension at 72°C for 10 min. The PCR reactions were performed in a MaxyGene Gradient (Foster City CA, USA). The PCR products were run on a 2% agarose gel containing 0.5 mg/ml ethidium bromide. Analyses and imaging of the gel were performed with the Transilluminator MultiDoc-Lt. Digital Imaging System (Upland CA, USA). The PCR products were sequenced in the ABI Prism 3130.
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Table 1. DNA samples from patients analyzed by PCR and sequencing.

<table>
<thead>
<tr>
<th>Species</th>
<th>Samples (n=60)</th>
<th>Samples analyzed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. furfur</em></td>
<td>12</td>
<td>12/60 (20)</td>
</tr>
<tr>
<td><em>M. globosa</em></td>
<td>10</td>
<td>10/60 (16.6)</td>
</tr>
<tr>
<td><em>M. sympodialis</em></td>
<td>4</td>
<td>4/60 (6.6)</td>
</tr>
<tr>
<td><em>M. restricta</em></td>
<td>2</td>
<td>2/60 (3.3)</td>
</tr>
<tr>
<td><em>M. slooffiae</em></td>
<td>1</td>
<td>1/60 (1.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
<td>29/60 (48.3)</td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
<td>31/60 (51.6)</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>60/60 (100)</td>
</tr>
</tbody>
</table>

*M. furfur* 26S rRNA gene (GenBank accession No. KF733799); *M. globosa* 26S rRNA gene (GenBank accession No. KF733801.1); *M. sympodialis* 26S rRNA gene (GenBank accession No. KF733798); *M. restricta* 26S rRNA gene (GenBank accession No. KF733797); *M. slooffiae* 26S rRNA gene (GenBank accession No. KF733800.1).

system (Applied Biosystems, Foster City CA). The positive strand sequences were analyzed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare with available Malassezia spp. sequences. The sequences obtained from five samples were submitted to Genbank.

Statistical analysis

A Chi-squared test was performed to evaluate the distribution differences of Malassezia species using SPSS v. 17.0 (Chicago, IL USA). P values of < 0.05 were considered significant.

RESULTS

Observation with the microscope allowed the detection of small cells, oval or ellipsoid, with the shape of a bottle or bowling pins, which is characteristic of this yeast.

Malassezia species identification

Of the 60 patient samples analyzed, 29 were positive for Malassezia, including 12 for *M. furfur* (20%), 10 for *M. globosa* (16.7%), 4 for *M. sympodialis* (6.7%). The lowest frequency values were for *M. restricta* and *M. slooffiae* with 2 (3.3%) and 1 (1.7%), respectively. Of the total samples, 51.6% (31/60) were negative for yeast (Table 1). Sequence alignments indicated that *M. furfur*, *M. sympodialis*, *M. restricta* and *M. slooffiae* isolates had 99% homology with the type strains. Only the *M. globosa* sequence had 100% homology with *M. globosa* (GenBank accession No KF733801.1) (Table 1).

Species distribution according to disease severity

The patients were divided into three groups according to the severity scale published by Peyri and Lleonart (2007) which evaluates the signs and symptoms of the disease, such as erythema, scaling, itching and infiltration. Moderate disease was the predominant clinical form in our population (47.6%), followed by severe (33.0%) and mild forms (18.3%). There was no significant difference in the species distribution among these groups (P > 0.05) (Figure 1). In the survey, 56.7% of the immediate family members were affected by SD (Figure 2). The father was the most affected family member (18.3%) (Figure 3). Outbreaks with emotional stress were experienced by 65.0% of patients.

DISCUSSION

SD is a chronic inflammatory skin condition and is a frequent cause of dermatology consults with an important impact on patients’ quality of life (Smith, 2001; Fivenson et al., 2002). The cause of seborrheic dermatitis is not completely understood. SD has an increased prevalence in patients using psychotropic medications and patients with Parkinson’s disease, other dermatoses (such as acne and rosacea), stress and AIDS (Jensen et al., 2000). One of the most studied causes is the association of colonization by the lipophilic yeast of the genus Malassezia. However, this association remains unclear. Yeast colonization in skin lesions varies from 45-100%. In our study, Malassezia was isolated in 48.3%, a value similar to that reported by Byung et al. (2010) in Korean patients. The controversy of the association of yeast with SD pathogenesis remains under discussion because Malassezia is not isolated in all cases. In recent years, there has been special interest in determining the distribution of Malassezia species in SD, and several studies have demonstrated important variations in the species isolated depending on geographic location. Some researchers have found predominantly *M. globosa* (Van Belkum et al., 1994; Rincón et al., 2005; Byung et al., 2010; Gaitanis et al., 2006; Gupta et al., 2001; Nakabayashi et al., 2000) while others *M. restricta* (Gemmer et al., 2002; Lee et al., 2001) or *M. sympodialis* (Falk et al., 2005). None of the studies reported *M. furfur* as the most common agent. To our knowledge, only Hernandez et al. (2003) has previously studied Mexican patients. In their study, of 15 cases, the most commonly
Figure 1. Distribution of Malassezia species according to severity of the disease. Moderate disease was most common in our population and *M. fur* was found in all severities.

Figure 2. Percentage of patients reporting any immediate family member affected or unaffected with SD. Most patients had some affected relatives (56.7%).

Figure 3. Detailed graph of family members reported with SD. The farthest was the most affected (28.3%).
isolated species was *M. sympodialis* (38.2%) followed by *M. furfur* (26.5%). These findings contrast with our study where the most frequently isolated species was *M. furfur* (20.0%) followed by *M. sympodialis* (16.7%) and *M. sympodialis* (6.7%), a correlation was not found between the severity of SD and species distribution. This result is similar to the data obtained by Prohic (2010), in a study of 40 patients where the species identification was performed by chemical methods. The most commonly isolated fungi were *M. restricta* (27.5%), followed by *M. globosa* (17.5%) and *M. slootia* (15%). However, the authors did not find a significant difference in the distribution of isolated Malassezia species according to the severity of scalp involvement. A study by Misery et al. (2007) found that 100% of the SD cases were associated with stressful conditions. In another study, the clinical characteristics of SD, therapeutic strategies employed in current clinical practice, and impact on quality of life were studied in a Spanish population. Two thousand-one-hundred and fifty nine patients were included. The mean age was 43.6 years; and 55% were men. In total, 42% had a family history of seborrheic dermatitis. Ninety-eight percent of patients reported trigger factors, such as stress/depression/fatigue (76%) and seasonal variation (44%) (Prohic, 2010; Dawson, 2007). In the present study, the majority (65%) of patients reported that stress was related to outbreaks. Another finding (not previously described) was the disparity between the prevalence of an affected father (20%) as compared to the mother (5%). This finding could suggest direct contact transmission that could be studied using subtyping methods or whole genome sequence comparisons.

**Conclusion**

This is the first study in Mexico that determined (by molecular methods) the presence and species distribution of Malassezia in the skin of patients with SD. *M. furfur* was the dominant species in our population and is in contrast with other studies. There was no significant association between the species isolated and disease severity. It was found that most patients mentioned a paternal relative with the same dermatosis. There are probably genetic or environmental factors that alter the ecosystem of saprophyte microorganisms in human skin, which could make individuals susceptible to this condition. Studies with a larger numbers of patients are necessary to confirm these findings.

**Conflict of interests**

The authors have not declared any conflict of interest.

**REFERENCES**