Identification of *Malassezia* Species Isolated from Patients with Pityriasis Versicolor Using PCR-RFLP Method in Markazi Province, Central Iran

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(Received 12 Oct 2013; accepted 15 Feb 2014)

**Abstract**

**Background:** The lipophilic yeasts of *Malassezia* species are members of the normal skin microbial that are cause of pityriasis versicolor. Pityriasis versicolor is a common superficial fungal infection with world-wide distribution. The phenotypic methods for identification of *Malassezia* species usually are time consuming and unreliable to differentiate newly identified species. But DNA-based techniques rapidly and accurately identified *Malassezia* species. The purpose of this study was isolation and identification of *Malassezia* Species from patients with pityriasis versicolor by molecular methods in Markazi Province, Central Iran in 2012.

**Methods:** Mycologic examinations including direct microscopy and culture were performed on clinical samples. DNA extraction was performed from colonies. The ITS1 region of rDNA from isolates of *Malassezia* species were amplified by PCR reaction. The PCR were digested by Cfo I enzyme.

**Results:** From 70 skin samples, were microscopically positive for *Malassezia* elements, 60 samples were grown on culture medium (85.7%). Using PCR–RFLP method, that was performed on 60 isolates, 37 (61.6%) *M. globosa*, 14 (23.3%) *M. furfur*, 5 (8.4%) *M. sympodialis* and 4 (6.7%) *M. restricta* were identified. In one case was isolated *M. globosa* along with *M. restricta*.

**Conclusion:** The PCR-RFLP method is a useful and reliable technique for identification of differentiation of *Malassezia* species.

**Keywords:** *Malassezia* spp., Pityriasis versicolor, Identification, PCR-RFLP, Iran

**Introduction**

*Malassezia* species are lipophilic yeasts which are as part of the normal flora of human skin, and are found in mostly of healthy adults. These yeasts are the cause of pityriasis versicolor and *Malassezia* folliculitis, and also implicated in the pathogenesis of common skin disorders such as seborrheic dermatitis, psoriasis, and atopic dermatitis (1). Pityriasis versicolor is a superficial fungal infection that usual clinical feature is slightly scaly patches of variable color (either pink, brown, or white) with irregular margin, most commonly found on the trunk and shoulders. These lesions different in...
color, and can be hypo- or hyperpigmented. The relationship between Malassezia species and their pathogenetic relationship was determined. In 1996, Guého et al. classified Malassezia genus into seven species, M. furfur, M. obtusa, M. globosa, M. slooffiae, M. sympodialis, M. pachydermatis, and M. restricta, based on morphological and biochemical characteristics (2). However, with the development of molecular methods such as polymerase chain reaction (PCR), additional five species have been described which are M. yamatoensis, M. nana, M. japonica, M. equi, and M. dermatis (3-8). However, these phenotypic methods are usually time consuming, lack sufficient discriminatory power, and are unable to unambiguously differentiate newly identified species. Various DNA-based molecular methods have been described to overcome this problem (9).

The aim of this study was to identify Malassezia species on their colonies, which were grown in culture media using 26S rDNA polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP).

Materials and Methods

Subjects
Patients with clinically suspected with pityriasis versicolor, referred to the Mycology Laboratory of the Arak University of Medical Sciences were recruited. Seventy patients (85.2%) were confirmed with pityriasis versicolor, based on the presence of both hyphae and yeasts in direct microscopy.

Collection and culture of sample
The samples were collected by scraping. The sampled skin was inoculated in modified Leeming and Notman culture medium (1% w/v peptone, 1% w/v glucose, 0.2% w/v yeast extract, 0.8% desiccated ox bile, 0.1% v/v glycerol, 0.05% w/v glycerol monostearate, 0.5% v/v Tween 60, and 2% v/v oleic acid, 1% w/v agar in distilled water) supplemented with cyclohexamide (0.5%) and chloramphenicol (0.05%), and was incubated at 32°C for two weeks. Isolated yeasts in 30% glycerol solution at -70 °C were stored in the freezer.

DNA extraction and 26S rDNA PCR
Genomic DNA was extracted using glass bead method (10). Primers were selected to allow the amplification of target DNA in all species. Their sequences were: forward, 5'-TAACAAGGAT-TCCCTAGTA-3' and reverse, 5'-ATTAC-GCCAGCATCCTAAG-3' (11). PCR amplification was performed in a final volume of 50 µl. Each reaction contained 1 µl of template DNA, 0.5 µM of each primer, 0.20 mM of each deoxynucleoside triphosphate (dNTPs), 5 µl of 10x PCR buffer, and 1.25 U of Taq polymerase. An initial denaturation step at 94 °C for 5 min was followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension step of 72 °C for 7 min. Amplified products were visualized by 1.5% (w/v) agarose gel electrophoresis in TBE buffer, stained with ethidium bromide (0.5 μg/ml), and photographed under UV transillumination.

RFLP analysis
The Cfo I enzyme (Roche Diagnostics, Mannheim, Germany) was used in this study (7). The restriction enzyme reaction was performed by incubating a 21.5 µl aliquot of PCR product with 10 U of the enzyme in a final reaction volume of 25 µl at 37 °C for 3 h. After the reaction, RFLP pattern was analyzed with DNA fragments in 2% agarose gel electrophoresis and staining with ethidium bromide.

Results

From 70 skin samples, which were microscopically positive for Malassezia elements, 60 samples were grown on culture medium (85.7%). Using PCR reaction to amplify the 26s rDNA region of all Malassezia species, 580 bp PCR bands were confirmed in all of isolates (Fig.1). RFLP analysis of PCR products of the ITS1 region with restriction enzyme Cfo I was undertaken on 60 isolates.

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Fig. 1: 26S rDNA PCR products before digestion with CfoI enzyme. Lanes 1-2: M. globosa, Lane 3: M. globosa&M. restricta, Lane 4: M. furfur, Lane 5: M. sympodialis, Lane 6: M. restricta, M: 50 bp ladder

Using enzyme CfoI (Roche Diagnostics, Mannheim, Germany), we could distinguish four different species, including 37 (61.6%) M. globosa, 14 (23.3%) M. furfur, 5 (8.4%) M. sympodialis and 4 (6.7%) M. restricta. In one case was isolated M. globosa along with M. restricta. In this study M. pachydermatis, M. obtusa, M. slooffiae and other Malassezia species were not isolated (Fig. 2).

Fig. 2: 26S rDNA PCR products after digestion with CfoI enzyme. Lanes 1-2: M. globosa, Lane 3: M. globosa&M. restricta, Lane 4: M. furfur, Lane 5: M. sympodialis, Lane 6: M. restricta, M: 50 bp ladder

Discussion

The Malassezia genus has undergone several taxonomic revisions since 1996 (2). Recently, on the basis of DNA relatedness, five new species have been included: M. dermatis, M. nana, M. japonica, M. yamatoensis, and M. aequi (3-8). Accurate and reproducible methods of species identification are essential for epidemiological purposes. Various molecular methods of characterizing species of Malassezia including Pulsed-field gel electrophoresis (PFGE) of Malassezia species, internal transcribed spacer 1 (ITS1) ribosomal DNA sequences, AFLP genotyping and random amplified polymorphic DNA (RAPD) have been used (12-14). But this methods are time consuming and too expensive for use as a routine diagnostic method. Recently, PCR-RFLP method used for identification of Malassezia species from colony and patient skin scales (11, 13, 15-17). Thus in this study, we used PCR-RFLP for identification of Malassezia species in Arak City, Iran.

In our study, 70 skin samples were microscopically positive for Malassezia elements and 60 samples were grown on culture medium (85.7%). The PCR–RFLP performed on 60 colonies and identified common species of Malassezia. The commonest of species was M. globosa, that was similar to other studies (11,17-24). However, Makimura et al., isolated M. furfur as the commonest species (9). In this study, in one case M. globosa along with M. restricta was isolated.

Among Malassezia species of human skin flora (25-27), M. sympodialis is the dominant species. Predominance of M. globosa from patients with pityriasis versicolor can be due to pathogenic characteristics of this species.

Conclusion

M. globosa was the commonest of Malassezia species isolated from patients with pityriasis versicolor in Arak City. This study provides important data for epidemiological status of Malassezia species in center of Iran.
Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

This project was supported by Arak University of Medical Sciences, Arak, Iran. The authors declare that there is no conflict of interests.

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