

## Molecular Identification of *Malassezia* Species with Direct DNA Extraction from Scalp of Patients with Dandruff and Seborrheic Dermatitis

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*Malassezia* genus in dandruff and seborrheic dermatitis which together have affected more than 50% of humans, have increasing proliferation in scalp. Proliferation of yeasts lead to scalp-flaking and create physical and mental disorder in people. The conventional culture-based methods for the isolation and identification of the *Malassezia* species in relative disease are labour intensive and time-consuming. We aimed to conduct a molecular analysis with direct DNA extraction from scalp in such patients at least time without complexity. In this cross sectional study, samples were taken from 65 patients with dandruff and seborrheic dermatitis. DNA extraction was performed directly from uncultured scalp by hexadecyltrimethylammonium bromide (CTAB method). Using species-specific primers derived from the 26S rDNA, PCR amplifications were performed. Identification of the species was carried out with enzyme CfoI in RFLP technique. CTAB method was applied as a more successful method for DNA extraction. *Malassezia restricta* was identified in %58.4 of the scalp specimens as the predominant species. In other 41.6% of cases, *Malassezia restricta* and *Malassezia globosa* were detected together. Molecular-based method of this study is able to diagnosis *Malassezia* yeasts in scalp without time-consuming culture-based method. Also the study shows the invasive characteristics of *Malassezia restricta* and *Malassezia globosa* species.

**Key words:** *Malassezia*, Dandruff, seborrheic dermatitis, CTAB, 26 S rDNA.

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*Malassezia* genus are the yeast that cause systemic disease like fungemia and are involved in other diseases such as dandruff, seborrheic dermatitis, onychomycosis, psoriasis, sinusitis, peritonitis, fungemia, blepharitis, folliculitis and inflammation of the lacrimal duct. They are budding-yeasts, often lipophilic and normal flora of humans skin and warm-blooded animals that under certain conditions increases their

proliferation rate (Zaini *et al.* 2013; Gemmer *et al.* 2002; Shokohi *et al.* 2008; Roberts 1969; Cafarchia *et al.* 2011;). In dandruff and seborrheic dermatitis (D/SD), that altogether have affected more than 50% of people, yeast proliferation and scalp-flaking increase and create physical and mental disorder, especially in youth which their health and hair hygiene and beauty is more important (Xu *et al.* 2007; Grimal 2007; Thomas and Dawson, 2007; Marcon and Powell, 1992; Ingham and Cunningham, 1993; Midgley, 2000; Ashbee and Evans, 2002; Thayikkannu, 2015).

Due to the multilayered cell wall of *Malassezia*, extraction of DNA is associated with

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problems (Mahmoudi Rad, 2011). In addition, DNA extraction directly from the scalp without time consuming culture method, needs the standardized method that can identify the species at least time . According to the last classification at least 14 species of *Malassezia* have been identified (Cafarchia et al. 2011), that are: *Malassezia furfur*, *Malassezia obtusa*, *Malassezia globosa*, *Malassezia slooffiae*, *Malassezia sympodialis*, *Malassezia pachydermatis*, *Malassezia restricta*, *Malassezia dermatis*, *Malassezia equina*, *Malassezia japonica*, *Malassezia nana*, *Malassezia yamatoensis*, *Malassezia caprae* and *Malassezia cunicoli*.

Many studies have been reported about seborrheic dermatitis and pityriasis versicolor that performed based on different molecular diagnosis (Shokohi, et al. 2008; Mahmoudi Rad, et al. 2011; Talaei, et al. 2014; Didehdar, et al. 2014; Velegraki, et al. 2015; Lee, et al. 2011; Zhang, et al. 2013). In such studies different DNA extraction methods and PCR from different regions have been performed. For molecular diagnosis, 26 S rDNA region that is protected area and varies between species of *Malassezia* genus, is suitable for the selection of primers to identifying of species in PCR-RFLP. Therefore, application of rapid molecular diagnosis method has a therapeutic importance that have been considered as the aim of this study. In the present study, for the first time in Iranian patients, identification of *Malassezia* species in scalp samples were carried out directly by CTAB DNA extraction.

## MATERIALS AND METHODS

### Study Subjects

In this cross sectional study, samples were taken from scalp of 65 patients with dandruff (19 patients) and seborrheic dermatitis (46 patients). The patients had been referred after visiting by specialist physician to the medical mycology laboratory of Tehran university of medical sciences for diagnosis the existence and role of *Malassezia* species in their disease, from February of 2014 to February of 2015. To ensuring the role of *Malassezia* species in causing of dandruff and seborrheic dermatitis, direct microscopic examination (DME) was performed and those samples with no yeast in DME were not

enrolled in this study. Also patients who had used any antifungal drug or antifungal shampoo at three days ago, were not enrolled in this study.

### Sample Collection

Scalpel was used to collect samples of scalp in the sterile plates by scraping. Using a standardized questionnaire, the patients were interviewed regarding age, gender, marital status, urban or rural resident, bathing interval and existence of sweating and stress. Patients who have stress and sweating were under surveillance of specialist physician. Minimum age of subjects was 11 years old and maximum age was 54 years old. 63% of the patients were celibate and 37% were married; 45% of the patients were urban residents and 55% were rural residents. 8% of patients have 1-2 days bathing interval; 18% with 3-4 days and 74% with more than 5 days bathing interval. The study was approved by the ethical committee of Tarbiat Modares university and all participants provided written informed consent to take part in this survey.

### Direct microscopic examination (DME)

Some of scrapings were transferred on a slide contains one drop sterile distilled water. A second slide was placed over it that the combination looked like a cross. Two smears were prepared by pressing the slides against each other. After dried in room temperature, they were heat fixed and stained with methylene blue. The direct microscopic examination (Olympus, Germany) of stained slides revealed different shapes - globular and oval - (Figure 1) of budding yeast cells with broadband or narrowband connections; furthermore, mycelium existence were surveyed too. Yeast quantification in scalp (dandruff) was done according to pattern that described before (Zareei, et al. 2013).

### DNA extraction

Effort was made to extraction of DNA from a tiny amount of skin scales collected through a noninvasive approach. Samples were suspended in 300 µl buffer [5% CTAB (Sigma); 700mM Tris-HCl, pH8; 10mM EDTA; and 5% 2-mercaptoethanol], Then, 300 µl glass beads (1.0 mm diameter) were added and samples were vortex mixed for 3 min. To the homogenate, 200 µl of CTAB buffer was added and following incubation for 30 min at 65 °C with occasional swirling, chloroform - isoamyl alcohol (vol : vol, 24 : 1)

extraction was performed. Following centrifugation the aqueous phase was obtained and nucleic acids were precipitated with cold isopropanol by centrifugation. DNA was washed in 70% ethanol to collect the nucleic acids by centrifugation as before. The pellet was dried and resuspended in 30–40 ml sterile double-distilled water.

#### PCR Reaction

Identification of *Malassezia* species was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP). Primer selection was based on alignment of published 26S rDNA sequences of known *Malassezia* species (accession numbers: AJ249950, AJ249951, AJ249952, AJ249953, AJ249954, AJ249955, AJ249956). PCR amplification from 26S rDNA fragment was carried out in 25 µl final volume using 10 pmole of forward, 5'-TAACAAGGATTC CCCTAGTA and reverse, 5'-ATTACGCCAGCAT CCTAAG primers. Each reaction contained 12.5 µl of PCR master mix, 2 µl of template DNA, 0.5 µM of each primer. An initial denaturation step at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step of 72 °C for 5 min (Eppendorf, Hamburg, Germany). Amplified products were visualized beside 50 bp ladder (Vivantis.Malaysia) by 1.5% (w/v) agarose gel electrophoresis in TBE buffer, stained with ethidium bromide (0.5 µg ml<sup>-1</sup>), and photographed under UV transillumination.

#### RFLP technique

The amplification of desired fragments (approximately 580 bp), the PCR products were digested by endonuclease CfoI enzyme (Thermo Scientific- Nedayfan Co- Iran). Digestion was performed by incubating a 17 µl aliquot of PCR product with 10 U of the enzyme in a final reaction volume of 20 µl at 37 °C for 3 h. The PCR products containing *Malassezia restricta* had no cleave and remained without change on first fragments( 580 bp), whereas PCR products containing *Malassezia globosa* were cleaved into two fragments (455 and 129 bp). The DNA fragments were separated and visualized beside 50 bp ladder (Vivantis.Malaysia) by electrophoresis on 2% agarose gel containing Green Viewer® dye or ethidium bromide.

#### Statistical analysis

The data analysis was performed by SPSS software (V.18). T-test was used to evaluate

statistical significance of the differences in comparative analyses. The study was assessed by using standard Chi-squared and 95 % Confidence intervals (CI). *p* value <0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

DNA extraction and PCR amplification of all samples were carried out successfully and products were detected on the gel; RFLP technique was also successfully performed in all specimens and species were identified (Figure 2). The prevalence of *Malassezia* species were: *Malassezia restricta* in 58.4% of cases was as a unique specie and in 41.6% of cases was mixed with *Malassezia globosa*. The Prevalence of species is the same in both sexes and in age groups as indicated in table 1.

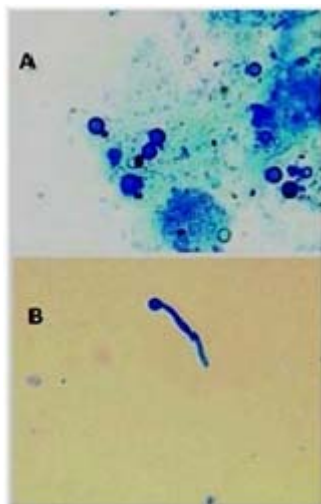
The highest amount of yeast in the patients scalp with microscopic examination were observed in the age group 21-30 among women and celibate people and rural residents, but there was no statistically significant relationship. Significant correlation was observed between the highest amount of yeast and people with stress (*P*=0.02) and sweating (*P*=0.03) (Tables 2 and 3). Between the amount of yeast and bathing interval, significant correlation was not observed (*P*= 0.5). In 16 cases (24.6%) in direct smears mycelium was observed that most of the cases (10 cases), it was found *Malassezia restricta*.

Many investigators and clinicians believe that *Malassezia* species play an important role in the causing of D/SD (Zaini et al. 2013; Gemmer et al. 2002; Shokohi et al. 2008). Confidential and non time-consuming methods for identification of agents have therapeutic importance. For sampling of patients with dandruff and seborrheic dermatitis, scraping with the scalpel was used in this study. The advantage of this method compared to other methods (Gupta and Kohli 2004; Hedayati et al. 2010), is to getting the proper amount of DNA extracted directly from scalp. In order to ensure yeast infection in scalp and estimate the amount of yeast and mycelium and their relationship to various factors, direct microscopic slides were prepared. Despite the severity of the yeast amount between the age group of 21- 30 years, there was no statistically significant differences observed

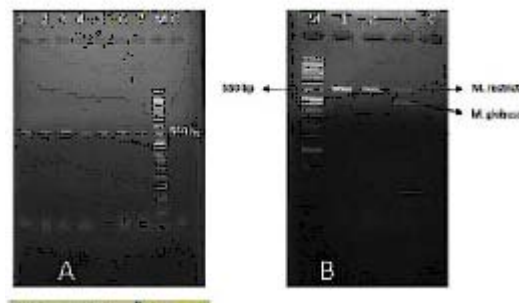
between the yeast quantity and age groups. This is consistent with the previous results of Lee et al. (2011) and the other studies (Mahmoudi Rad et al. 2011; Hedayati et al. 2010). It seems that severity of the yeast amount can be due to age range which sebaceous glands are very active and secretive of sebum.

There was no significant relationship between the yeast quantity and genus (Table 4). It

seems that differences in sexual hormones do not related to selective use of sebum composition for yeast growing. This result is in accordance with the study of Lee and colleagues in Korea (2011) and Zhang et al. in China (2013). Due to the lack of a significant relationship between the place of residence and the yeast quantity, this can reflect that difference in the level of health care in rural areas and the urban areas is low.



**Fig. 1.** Section A is DME of globular budding yeast cells that were observed in scalp. Section B is DME of oval cell with mycelium that was observed in scalp



**Fig. 2. A:** lanes 1-7 are 580 bp fragment of *Malassezia* amplified by universal primers in PCR. lane M is 50 bp ladder and lane C is Negative control that were observed by electrophoresis on 1.5% agarose gel.

**B:** lane M is 50 bp ladder. lanes 1 and 2 are *M. restricta*. lane 3 is *M. restricta* and *M. globosa* as mixed infection and lane C is Negative control that were observed by electrophoresis on 2% agarose gel.

**Table 1.** Frequency of *Malassezia* species in scalp of patients with different age groups

Age group	Species prevalence		Total	P-value, df (95 % CI)
	<i>M. restricta</i>	<i>M. restricta</i> + <i>M. globosa</i>		
11- 20	12(18%)	13(20%)	25(38%)	0.5 , 3
21- 30	22(34%)	11(17%)	33(51%)	
31- 40	3(5%)	2(3%)	5(8%)	
> 40	1(1.5%)	1(1.5%)	2(3%)	
Total	38(58.5%)	27(41.5%)	65(100%)	

**Table 2.** Effect of sweating on yeast proliferation in scalp of patients with dandruff and seborrheic dermatitis

P-value, df (95 % CI)	Total	Yeast quantity			Sweat	Yes
		severe	moderate	weak		
0.03 , 2	39(60%)	25(39%)	4(6%)	10(15%)	No	
	26(40%)	16(24%)	8(12.5%)	2(3.5%)		
	65(100%)	41(63%)	12(18.5%)	12(18.5%)	Total	

Significant correlation was observed between stress ( $P=0.02$ ) and sweating ( $P=0.03$ ) with yeast quantity, (Tables 2 and 3). Stress cause secretion of sweat glands and sweating, moisturize the skin and prevent normal turning over of skin cells, favoring the growth and proliferation of the yeast on the skin. As our medical mycology laboratory that these result were obtained is a reference laboratory; these finding can be generalize to the society. Thus in treatment of patient with dandruff and seborrheic dermatitis, these predisposing factors must be considered. A significant relationship between the yeast quantity with bathing interval was not observed; it seems whatever causes severe yeast proliferation, is sebum secretion and its composition (Takahata et al. 2007). Anything that increases the secretion of sebum, will cause proliferation and desquamation of the skin.

Mycelium was observed in 24.6% of cases in direct smear. In most cases *Malassezia restricta* was the only detected agent. This finding suggests that this species also has the ability to produce mycelium as *Malassezia furfur* and *Malassezia globosa*; although in Morishita et al. study in Japan *Malassezia globosa* has been reported as only mycelium producing species (Morishita et al. 2006). There was no statistically significant differences between the production of mycelium

with factors such as age ( $P= 0.2$ ), genus ( $P= 0.2$ ), sweating ( $P= 0.3$ ), stress ( $P= 0.2$ ) and yeast quantity, suggests that production of mycelium can be due to invasive characteristics of the yeast.

To getting the proper amount of DNA extracted directly from scalp, for DNA extraction, combination of two mechanical and chemical methods was used. In mechanical method, glass bead and in chemical method, CTAB were used. This method was very successful. In 100% of cases, fungal cells DNA were obtained. In addition of method that applied in this study, perhaps the other reason of this success is the sampling method that performed by scraping of scalp to getting the proper amount of DNA extracted directly from scalp. CTAB lysis buffer without mechanical process were used by Gaitanis previously and its success and sensitivity was 50% (Gaitanis et al. 2002). In addition, in their study, to amplify target fragment, nested PCR method has been used. In our study, combination of mechanical and chemical methods improved the success. This method is compatible with the successful methods that were described previously (Zhang et al. 2013; Morishita et al. 2006; Tajima et al. 2008;). The highest extracted DNA was obtained by Tajima et al. (2008). They performed extraction using liquid nitrogen and boiling. The acrylamide polymer and glycogen were used to precipitate DNA. In Morishita et al. (2008)

**Table 3.** Effect of stress on yeast proliferation in scalp of patients with dandruff and seborrheic dermatitis

P-value, df (95 % CI)	Total	Yeast quantity			
		severe	moderate	weak	
0.02, 2	32(49%)	25(39%)	5(7.5%)	2(3.5%)	Stress Yes
	33(51%)	16(24%)	7(12%)	10(15%)	
	65(100%)	41(63%)	12(18.5%)	12(18.5%)	Total

**Table 4.** Effect of genus on yeast proliferation in scalp of patients with dandruff and seborrheic dermatitis

P-value, df (95 % CI)	Total	Yeast quantity			
		severe	moderate	weak	
0.1 , 2	33(51%)	18(28%)	9(13.75%)	6(9.25%)	Genus Male
	32(49%)	25(35%)	3(4.25%)	6(9.25%)	
	65(100%)	41(63%)	12(18.5%)	12(18.5%)	Total



and Zhang et al. (2013) studies, for amplification of target, species, specific primers and nested PCR method have been used. Thus, as were described above, method of this study can be used as a successful method for DNA extraction directly from scalp in diagnosis laboratory and research centers with non time-consuming and without complexity advantage .

To diagnosis and identifying of species, 26 S rDNA fragment was selected. This region is protected area in genus *Malassezia* for the selection of universal primers and varies between species. Using *CfoI* restriction enzyme, 9 species were distinguished, firstly described by Mirhendi et al. (2005) and then have been used in others study (Lee et al. 2011; Oh et al. 2010; Jang et al. 2009; Oh et al. 2009;).

Due to limited of sampling to the scalp, two species *Malassezia restricta* and *Malassezia globosa* were identified. *Malassezia restricta* in 58.4% of cases was as unique agent of infection and in 41.6% of cases was mixed with *Malassezia globosa*. This result is somewhat in accordance with other studies such as: Prohic et al. study in Bosnia (2010) on the scalp that only three species *Malassezia restricta*, *Malassezia globosa* and *Malassezia slooffiae* were identified.

In Oh study in Korean (2010) in patients with SD, after culturing of samples and DNA extraction from isolated yeast colonies, *Malassezia restricta* was identified as the prevalent species. In the Gemmer et al. study (2002) that *Malassezia* genome was extracted directly from the scalp of the dandruff sufferers, *Malassezia restricta* and *Malassezia globosa* were identified. In Lee et al. study (2011) in patients with SD, *Malassezia restricta* in 47% and *Malassezia globosa* in 27% of cases were the predominant species which identified after the culturing by molecular methods. In their study 15% of cases were mixed infections with mixed species. In Zhang et al. study in China (2013) in patients with SD, *Malassezia globosa* and *Malassezia restricta* were reported as predominant species in 87% and 81% of cases, respectively; that DNA was extracted directly from the scalp, in their study, 83% of cases were infected with mixed species even up to 4 species. In Jang et al. study in Korea (2009) after the identification of culture colony by molecular method, the most

common species in the scalp were *Malassezia restricta* (57%), *Malassezia globosa* (22%) and *Malassezia sympodialis*.

The prevalence of species in our study is not in accordance with Shokohi et al. study (2008) (in order of prevalence: *Malassezia furfur*, *Malassezia globosa* and *Malassezia restricta* ) and Zarei et al. study (2013) (in order of prevalence: *Malassezia globosa*, *Malassezia pachydermatis*, *Malassezia furfur* and *Malassezia restricta*), in these studies organism have been identified after culture. In the former with molecular and in the latter with biochemical method, species have been identified and mixed infection with mixed species have not been reported. Possible causes of these differences might be subsequent to the growth rate of the species cultured on the medium, the faster growing species colonies covered slow growing species colonies. During the removing of the colony to differential tests, only surface colony are removed and identified; this point is true especially about *Malassezia restricta* which grows so slow and is very sensitive to environmental conditions (Shokohi et al. 2008; Morishita et al. 2006). Also, in catalase differential test, if *Malassezia restricta* be mixed with other species, is not diagnosed. It can be said that in the methods based on direct extraction of DNA from samples toward culture-based methods, more species are isolated (Lee et al. 2011) and *Malassezia restricta* is the predominant and prevalent species (Gemmer et al. 2002; Morishita et al. 2006; Tajima et al. 2008; Sugita et al. 2001), but probably in culture-based methods *Malassezia restricta* do not grow or not identified (Gemmer et al. 2002; Tajima et al. 2008).

## CONCLUSION

Method of this study can be used as a successful method for DNA extraction from scalp in laboratory for diagnosis. Also mixed species in mixed infections can be better detected. The study shows the invasive characteristics of *Malassezia restricta* and *Malassezia globosa* species and predisposing factors such as sweating and stress in proliferation of yeast in scalp that must be considered in therapeutic aims of dandruff and seborrheic dermatitis .

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