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The Prevalence and Species Composition of *Malassezia* yeasts in Patients with Clinically Suspected Onychomycosis

Asja Prohic[1], Suada Kuskunovic-Vlahovljak[2], Tamara Jovovic Sadikovic[1], Semra Cavaljuga[3]

[1]Department of Dermatovenerology, University Clinical Center of Sarajevo, Sarajevo, Bosnia and Herzegovina
[2]Institute of Pathology, Faculty of Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
[3] Department of Epidemiology and Biostatistics, Faculty of Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

Corresponding author: Professor Asja Prohic, MD, PhD. Department of Dermatovenerology, University Clinical Center of Sarajevo, Bolnicka 25. 71000 Sarajevo, Bosnia and Herzegovina. Tel. +387 61 100 380, Fax. +387 33 297 801. e-mail: asjaprohic@hotmail.com

ABSTRACT

Introduction: There are limited numbers of studies which focused on the identification of *Malassezia* yeasts to a species level in onychomycosis. Therefore, the aim of our study was to determine the prevalence and species composition of *Malassezia* yeasts in patients with clinically suspected onychomycosis and to examine if the range of species varies with patient gender, age, site of involvement and clinical pattern of onychomycosis. **Methods:** Specimens were taken from 785 patients presenting signs of onychomycosis and then incubated on Sabouraud dextrose agar and modified Dixon agar. The yeasts isolated were identified according to their macroscopic and microscopic features and physiological characteristics. **Results:** *Malassezia* species were diagnosed both by microscopy and culture in fourteen (1.8%) patients. *M. globosa* was the predominant, if not only, species identified from nail samples. Mixed cultures were observed in five cases: in 4 cases *Malassezia* was co-isolated with *Candida albicans* and in one case with dermatophyte. Fingernails were affected more frequently than toenails (85.7%) and distolateral subungual onychomycosis was the most common clinical type (78.6%). **Conclusion:** No significant differences were found in the distribution of *Malassezia* species isolated according to demographic parameters.
Key words: *Malassezia* species, isolation, onychomycosis.

1. INTRODUCTION

Malassezia are lipophilic yeasts colonizing the skin of humans and in many warm-blooded animals. In contrast to their commensal roles, these organisms have been implicated in the pathogenesis of various dermatological disorders, including pityriasis versicolor (PV), *Malassezia* folliculitis, seborrheic dermatitis (SD), atopic dermatitis and psoriasis (1, 2). Additionally, different reports in the literature have linked *Malassezia* yeasts with cases of nail infection (3-10).

At present, fourteen *Malassezia* species have been identified from human (*M. dermatis, M. japonica* and *M. yamotensis*) and animal skin (*M. nana, M. caprae, M. equina* and *M. cuniculi*) (11). A large number of studies have been carried out world-wide on the distribution of *Malassezia* species on healthy and diseased human skin in which variable results have been reported from different geographical regions (3, 12-19). These variations are likely to have occurred due to different isolation techniques, different media and the various growth characteristics of each species (12).

The etiologic role of *Malassezia* species in onychomycosis is uncertain because these yeasts have not been shown to degrade keratin, an ability that is necessary to invade the nail.

Moreover, less is known about which of the currently recognized *Malassezia* species is linked to onychomycosis. The present study was designed to determine the prevalence and species composition of *Malassezia* yeasts in patients with clinically suspected onychomycosis and to examine if the range of species varies according to demographic parameters.

2. METHODS

Study Population

From 2000 to 2013 clinical specimens from 785 patients with clinically suspected onychomycosis referred to the Department of Dermatovenerology Sarajevo University Clinical Center were examined.

Only those subjects who had not used any topical and oral treatment or ultraviolet photo therapy in previous

two months were included in the study. No concomitant diseases were registered.

The Hospital Ethics Committee approved the study and all participants signed a written informed consent.

Samples

Samples of nail clippings and scrapings, including some debris from a nail bed, were collected with sterile surgical blades after cleaning the site with 70% ethanol.

A part of this material was examined microscopically after the treatment with 30% solution of potassium hydroxide and Parker blue ink; the rest was cultured into Sabouraud dextrose agar and into modified Dixon agar consisting of 3.6% malt extract, 0.6% mycological peptone, 2.0% desiccated ox bile (Sigma Chemical Co. Ltd, Dorset, UK), 1% Tween 40, 0.2% glycerol, 0.2% oleic acid, 0.05% chloramphenicol, 0.05% cycloheximide, and 1.2% agar pH 6.0. The medium was always used within one week of preparation and the cultures were inoculated at $32^{\circ}C$ for seven days.

Identification of Malassezia yeasts

Malassezia species were identified according to their macroscopic and microscopic features and physiological characteristics.

The macroscopic features of the predominant colonies included their shape, size, color, consistency, and the characteristics of medium around them.

Microscopic features of the yeast cells in culture were described after lactophenol staining and included the predominant morphology, size and budding base of the yeasts.

To assess the physiological properties of the yeasts catalase reaction was determined by using a drop of hydrogen peroxide (30%) onto a culture smear on a glass slide. The production of gas bubbles, indicative of release of oxygen, was considered a positive reaction. Utilization of Tween compounds was done according to the test originally described by Guillot *et al.* (20) and later modified by Gupta *et al.* (21). Yeast suspension, obtained by inoculating 5 mL of sterile water with a loopful of actively growing yeasts, was inoculated on Sabouraud glucose agar. The inoculum was then spread evenly. Each plate was divided into four sections and 5 mL of Tween 20, 40, 60 and 80 were added into a hole made in center of each section and incubated for a week at $32^{\circ}C$. Utilization of Tweens was assessed by the degree of growth and/or reaction of the lipophilic yeasts around individual holes.

The ability of the various *Malassezia* species to growth on mDA at $38^{\circ}C$ was studied. A sample of actively growing cultures was transferred to mDA and incubated on $38^{\circ}C$ for seven days after which the ability to grow was investigated.

The β-glucosidase activity (spliting of esculin) of different *Malassezia* species was assayed using method described by Mayser *et al.* (22). Briefly, a loop of fresh yeast was inoculated deeply in the esculin agar tube and incubated for 5 days at $32^{\circ}C$. The splitting of esculin is revealed by darkening of the medium.

Statistics

Chi-squared test with Yates' correction for a small sample size was carried out to determine the statistical significance of differences in proportions. We used a statistical software pacage Minitab 13.0. Significance level was set at $P < 0.05$.

3. RESULTS

Malassezia species were diagnosed both by microscopy and culture in fourteen (1.8%) cases. Only one *Malassezia* species was recovered from each of the samples.

The microscopic examination of samples showed the presence of yeast cells in 14 (1.8%) patients. Typical large and spherical yeasts producing buds on narrow base, which are identical in appearance to those of *M. globosa*, dominated; they were found in 8 (57.1%) cases. Smaller yeast cells, oval or cylindrical, with buds forming on narrow base, were seen in remaining 6 slides (42.9%), suggesting different *Malassezia* species. Short filaments were not observed in any sample.

Growth of *Malassezia* cultures was observed from all microscopically positive samples. Four species were isolated: the most common species was *M. globosa* (8; 57.1%), followed in frequency by *M. sympodialis* (3; 28.6%), *M. restricta* (2; 14.3%) and *M. furfur* (1; 7.1%).

Malassezia was isolated by culture as the only fungus in nine (64.3%) cases; in remaining five cases *Malassezia* was co-isolated with *Candida albicans* in four cases (28.6%) and in one case with dermatophyte (7.1%).

Distribution of Malassezia species isolated according to demographic parameters

Gender

Men and women were equally affected. No statistically significant differences were found between the genders in the species isolated (p>0.05).

species	Gender		Age groups					Site of involvement		Clinical pattern		
	F	M	0-15	16-30	31-45	46-60	>60	FN	TN	DLSO	PSO	TDO
M. globosa	5	3	0	1	1	5	1	7	1	6	1	1
M. sympodialis	1	2	0	0	0	2	1	2	1	2	1	0
M. restricta	1	1	0	0	1	1	0	2	0	2	0	0
M. furfur	0	1	0	0	0	0	1	1	0	1	0	0
TOTAL	7	7	0	1	2	8	3	12	2	11	2	1

Table 1. Malassezia species distribution from patients with clinically suspected onyhomycosis according to age, gender, site of involvement and clinical pattern. FN; Fingernails, TN; Toenails, DLSO, Disto–Lateral Subungal Onychomycosis; PSO, Proximal Subungual Onychomycosis; TDO, Total Dystrophyic Onychomycosis

Age

Patients were aged between 22 and 79 years (average 52.8 years). According to their age, they were divided into five groups as follows: less than 15 years (n=0; 0%), 16-30 years (1; 7.1%), 31-45 years (n=2; 14.3%), 46-60 years (n=8; 57.1%) and more than 60 years (n=3; 21.4%). No statistically significant differences were found in the species isolated in these five groups (p>0.05).

Site of involvement

Fingernails were affected in twelve of fourteen patients (85.7%) and alterations on both the fingernails and toenails in a single patient were not seen.

We found no statistically significant difference in the distribution of isolated *Malassezia* yeasts according to the site of involvement (p>0.05).

Clinical pattern of onychomycosis

The infection presented in eleven patients as disto-lateral subungual onychomycosis (DLSO) (78.6%), in two patients as proximal subungual onychomycosis (PSO) (14.3%), while total dystrophyic onychomycosis (TDO) was noted in one patient (7.1%).

There was no statistically significant difference in the distribution of isolated species according to clinical type of onychomycosis (p>0.05).

Distribution of *Malassezia* species isolated from patients with clinically suspected onyhomycosis according to demographic parameters is demonstrated in Table 1.

4. DISCUSSION

Malassezia yeasts, the recognized etiological agent of PV, has occasionally been reported as an agent of onychomycosis, although it may exist as a colonizer of subungual debris of patients with nail infections (23). Risk factors that could affect nail plate, such as nail trauma and psoriasis, are considered to be important in the development of *Malassezia* onychomycosis (3-5).

With the exception of *M. pachydermatis*, all of the *Malassezia* species are obligatory lipid-dependent and show an absolute requirement for lipids in culture media. This property influences their distribution in sebum rich areas of the skin such as scalp, face and the trunk (1, 23). Since a source of lipids is essential for their pathogenicity, the etiologic role of *Malassezia* in onychomycosis is speculative because these yeasts do not posses keratolytic properties and have no ability to invade nails, as they are not good source of lipids.

In our study *Malassezia* was isolated in 1.8% of the patients diagnosed with onychomycosis, alone in 64.3% of cases, but was associated with *Candida albicans* in 28.6% of cases which is in concordance with study of Escobar *et al.* (5) who found an association between two genera in 30%. Similarly, in a study conducted in Brasil, *Malassezia* yeasts were isolated in 3.8% of patients with clinically suspected onychomycosis, but in 3 cases co-isolated with *Candida albicans* or *Trichophyton rubrum* (4). Furthermore, Crespo-Erchiga *et al.* claimed that they co-isolated *Malassezia* yeasts with *Candida* as subungual flora members. Thus, because of its low incidence in the subungual debris, the authors could not substantiate that *Malassezia* is a pathogenic agent in onychomycosis (24).

We assume from these observations that initially the etiologic agent might have been a dermatophyte or yeast and that the nail damage caused by the supposed fungus might have promote the consecutive invasion by *Malassezia* yeasts. How long they can survive as a colonizer of subungual debris without lipids necessary for their growth, and whether their presence affects the clinical picture of the nail, remains to be established.

To date, the most common species cultured from healthy and diseased human skin are *M. globosa*, *M. sympodialis* and *M. restricta. M. sympodialis* emerges as the predominant species on healthy skin, especially on the trunk, where it can be recovered in great numbers in more than 40% of individuals (2, 12-14), while *M. globosa* is reported to be a causative agent of PV, found in filamentous form in the scales from this skin disorder (2, 14-16). In our study, *M. globosa* was the major isolate found in more than a half of all isolate (57.1%) and *M. sympodialis* was the second most common agent (28.6%). The remaining two species, *M. restricta* and *M. furfur*, were recovered in lower frequencies, 14.4% and 7.1%, respectively. *M. restricta* is isolated regularly from the scalp and face of healthy subjects and of patients with SD (2, 17-19), while *M. furfur*, besides colonizing the skin, also may cause systemic fungal infection, usually in neonatal and adult patients who are receiving parenteral nutrition (25, 26). Contrary to our findings, studies that have attempted to identify *Malassezia* to a species level, reported isolation of *M. furfur* from fingernails (6, 8-10) and/or from toenails (6, 7). However, all these studies reported single cases, in contrast to our study, which included fourteen cases of *Malassezia*-associated onychomycosis over a longer period.

The factors permitting the transition of the yeast to the mycelial phase are still little known but the production of various enzymes such as lipase, phospholipase and lipoxygenase could contribute to the pathogenic activity of these yeasts (27). The hyphal stage is considered to play a role in the pathogenesis of PV, as hyphae are abundantly demonstrable in scales from lesions (2, 14, 15). No evidence of mycelial phase in our study further supports the proposal that *Malassezia* yeasts are rather colonizer than etiological agents of onychomycosis.

Chowdhary *et al.* reported a case of onychomycosis in which the nail invasion of *Malassezia* was identified by conventional and molecular techniques and additionally was proven by histopathology. In addition, other concomitant diseases that could alter the nail plate were excluded (6). Furthermore, no comorbidities were registered in our study, contrary to the study of Zawar and Chuh (8) which reported a patients having PV with clinical and mycological evidence of fingernail infection related to *Malassezia*. The results of the present study showed that *Malassezia* onychomycosis was more frequent in fingernails, what is in concordance with some aforementioned studies (6, 8-10). Scratching, regardless of whether the skin is clinically healthy or in the presence of certain skin disorders, can lead to the transmission of *Malassezia* fungi from the skin where they reside as commensals or pathogens, to the nails, colonizing subungual debris. We thus postulate that these species were isolated from the nail samples as a part of the normal cutaneous micro flora. Moreover, the

presence of *Malassezia* yeast in subungual debris of fingernails would be relevant as a source of systemic infections particularly in the context of colonization of patients in hospital care units (25, 26).

Clinically, onychomycosis is classified into various types, of which Disto–Lateral Subungal Onychomycosis (DLSO) was found to be the commonest clinical pattern (28). The present study showed that DLSO was the commonest clinical pattern which was seen in 78.6% patients. Proximal Subungual Onychomycosis (PSO), noted in two cases, is considered extremely rare, being described in the literature in a single case (10).

Overall, onychomycosis is more common in adults than children and its prevalence increases with age (28, 29). In the present study, the majority of patients (57.1%) were above 46 years of age (78.6%) and the age group 46-60 contained the highest prevalence of *Malassezia* onychomycosis (57.1%). This finding is somehow in accordance with studies examining onychomycosis caused by other fungi (dermatophytes, yeasts and molds), as no relevant studies regarding *Malassezia* onychomycosis were found in the literature.

To conclude, no evidence of mycelial phase supports the proposal that *Malassezia* yeasts are rather colonizer than etiological agents of onychomycosis. Whether these yeasts are capable of inducing clinical symptoms of onychomycosis remains to be established. However, their presence in nail material is important as a possible source of systemic infections.

CONFLICT OF INTEREST: NON DECLARED.

REFERENCES

1. Gaitanis G, Velegraki A, Mayser P, Bassukas ID. Skin diseases associated with Malassezia yeasts: facts and controversies. Clin Dermatol. 2013; 31(4): 455-463.
2. Crespo Erchiga V, Ojeda Martos AA, Vera Casaño A, Crespo Erchiga A, Sánchez Fajardo F. Isolation and identification of Malassezia spp. in pytiriasis versicolor, seborrheic dermatitis and healthy skin. Rev Iberoam Micol. 1999; 16: 16-21.
3. Crozier WJ, Wise KA. Onychomycosis due to Pityrosporum. Australas J Dermatol. 1993; 34(3): 109-112.
4. Silva V, Moreno GA, Zaror L, de-Oliveira E, Fischman O. Isolation of Malassezia furfur from patients with onychomycosis. J Med Vet Mycol. 1997; 35(1): 73-74.
5. Escobar ML, Carmona-Fonseca J, Santamaría L. Onychomycosis due to Malassezia. Rev Iberoam Micol. 1999; 16(4): 225-229.
6. Chowdhary A, Randhawa HS, Sharma S, Brandt ME, Kumar S. Malassezia furfur in a case of onychomycosis: colonizer or etiologic agent? Med Mycol. 2005; 43(1): 87-90.
7. Ertam I, Aytimur D, Alper S. Malassezia furfur onychomycosis in an immunosuppressed liver transplant recipient. Indian J Dermatol Venereol Leprol. 2007; 73(6): 425-426.
8. Zawar V, Chuh A. Case report on Malassezia infection of palms and fingernails – speculations on cause for therapeutic failure in pityriasis versicolor. J Eur Acad Dermatol Venereol. 2009; 23(2) :171-172.
9. Zhao Y, Li L, Wang JJ, Kang KF, Zhang QQ. Cutaneous malasseziasis: four case reports of atypical dermatitis and onychomycosis caused by Malassezia. Int J Dermatol. 2010; 49(2): 141-145.
10. Zareei M, Zibafar E, Daie Ghazvini R, Geramishoar M, Borjian Borujeni Z, Hossein Pour L, et al. Proximal onychomycosis due to Malassezia furfur: a case report. Teheran Univ Med J (TUMJ). 2013; 70(12): 802-806.
11. Cabañes FJ. Malassezia yeasts: how many species infect humans and animals? PLoS Pathog. 2014; 10: e1003892.
12. Sandström Falk MH, Tengvall Linder M, Johansson C, et al. The prevalence of Malassezia yeasts in patients with atopic dermatitis, seborrhoeic dermatitis and healthy controls. Acta Derm Venereol. 2005; 85(1): 17-23.
13. Oh BH, Song YC, Lee YW, Choe YB, Ahn KJ. Comparison of Nested PCR and RFLP for Identification and Classification of Malassezia Yeasts from Healthy Human Skin. Ann Dermatol. 2009; 21(4): 352-357.
14. Kaur M, Narang T, Bala M, Gupte S, Aggarwal P, Manhas A. Study of the distribution of Malassezia species in patients with pityriasis versicolor and healthy individuals in Tertiary Care Hospital, Punjab. Indian J Med Microbiol. 2013; 31(3): 270-274.
15. Prohic A, Ozegovic L. Malassezia species isolated from lesional and non-lesional skin in patients with pityriasis versicolor. Mycoses. 2007; 50(1): 58-63.
16. Saad M, Sugita T, Saeed H, Ahmed A. Molecular epidemiology of Malassezia globosa and Malassezia restricta in Sudanese patients with pityriasis versicolor. Mycopathologia. 2013; 175(1-2): 69-74.
17. Gemmer CM, DeAngelis YM, Theelen B, Boekhout T, Dawson Jr TL Jr. Fast, noninvasive method for molecular detection and differentiation of Malassezia yeast species on human skin and application of the method to dandruff microbiology. J Clin Microbiol. 2002; 40(9): 3350-2257.
18. Prohic A. Distribution of Malassezia species in seborrhoeic dermatitis: correlation with patients' cellular immune status. Mycoses. 2010; 53(4): 344-349.
19. Lee YW, Byun HJ, Kim BJ, al. Distribution of Malassezia species on the scalp in korean seborrheic dermatitis patients. Ann Dermatol. 2011; 23(2): 156-161.
20. Guillot J, Gueho E, Lesourd M, Midgley G, Chevrier G, Dupont B. Identification of Malassezia species. A ppractical approach. J Mycol Med. 1996; 6: 103-110.
21. Gupta AK, Kohli Y, Summerbell RC, Faergemann J. Quantitative culture of Malassezia species from different body site of individuals with and without dermatoses. Med Mycol. 2001; 39(3): 243-251.
22. Mayser P, Haze P, Papavassilis C, Pickel M, Gruender K, Gueho E. Differentiation of *Malassezia* species: selectivity of Cremophor EL, castor oil and ricinoleic acid for *M. furfur.* Br J Dermatol. 1997; 137(2): 208–213.
23. Midgley G. The lipophilic yeasts: state of the art and prospects. Med Mycol. 2000; 38: 9-16.
24. Crespo Erchiga V, Delgado Florencio V. Malassezia species in skin diseases. Curr Opin Infect Dis. 2002; 15(2): 133-142.
25. Iatta R, Cafarchia C, Cuna T, et al. Bloodstream infections by Malassezia and Candida species in critical care patients. Med Mycol. 2014; 52(3): 264-269.
26. Shparago NI, Bruno PP, Bennett J. Systemic Malassezia furfur infection in an adult receiving total parenteral nutrition. J Am Osteopath Assoc. 1995; 95(6): 375-377.
27. Juntachai W, Oura T, Murayama SY, Kajiwara S. The lipolytic enzymes activities of Malassezia species. Med Mycol. 2009; 47(5): 477-484.
28. Lone R, Bashir D, Ahmad S, Syed A, Khurshid S. A study on clinico-mycological profile, aetiological agents and diagnosis of onychomycosis at a government medical college hospital in kashmir. J Clin Diagn Res. 2013; 7(9): 1983-1985.
29. Aghamirian MR, Ghiasian SA. Onychomycosis in Iran: epidemiology, causative agents and clinical features. Nihon Ishinkin Gakkai Zasshi. 2010; 51(1): 23-29.