

IDENTIFICATION AND PATHOGENICITY OF *MALASSEZIA* SPECIES ISOLATED FROM HUMAN HEALTHY SKIN AND WITH MACULES

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SHORT COMMUNICATION

ABSTRACT

This study investigated the occurrence of *Malassezia* species in clinically healthy students and with macules with a slight fawn discoloration and characterized the isolates as to the pathogenicity factors such as growth at 37°C, lipase, phospholipase and protease detection. Clinical samples were collected from different body sites of one hundred students of different ages and both sexes. The samples, obtained by scrapping the skin surface and the scalp, were treated with potassa and cultured. Cultures were obtained in Petri dishes containing Sabouraud agar medium added of olive oil, incubated at room temperature and at 37°C. Culture identifications were based in their morphological and physiological properties. Lipase, phospholipase and protease detection was performed in specific media on Petri dishes for formation of a zone. Globose, spherical yeast cells and hypha were investigated by direct microscopy of clinical materials. *Malassezia furfur* was detected in seven samples and *M. sympodialis* in four. All *Malassezia* cultures presented lipase activity, but none was phospholipase positive. Protease activity was observed in two *M. furfur* and two *M. sympodialis* isolates.

Key words: *Malassezia furfur*, *M. sympodialis*, pathogenicity

Malassezia is a lipophilic yeast frequently found in normal human skin. Current evidence indicates a high rate of skin colonization in healthy adults, in contrast with the low rate of colonization in prepubertal children (20).

Gueho *et al.* (11), recognized seven distinct species within this genus, namely *M. furfur*, *M. pachydermatis*, *M. globosa*, *M. obtuse*, *M. restricta*, *M. slooffiae* and *M. sympodialis*. Furthermore, recently three new species were included in this genus, namely *M. dermatis*, *M. yamatoensis* and *M. nana* (14,17,35,38). With the exception of *M. pachydermatis*, the remaining eight species have an absolute *in vitro* requirement for supplementation of long-chain fatty acids. Based on studies conducted within the last 5 years, it appears that the most frequently organism associated with pityriasis versicolor may not be *M. furfur* and possible candidates

include *M. globosa* (2,27) and *M. sympodialis* (15). The genus *Malassezia* was recently revised, but the clinical significance of each of these species is not clearly understood (15).

Pityriasis versicolor (tinea versicolor) is a superficial fungal infection of the skin, appearing most commonly on the upper trunk, as well as on the upper arms, neck and face. As suggested by its name, pityriasis versicolor may be manifested in different colours, ranging from pink or to dark brown or even black. The lesions have a characteristic flaking appearance, although in larger lesions this can be evident only at the border of the macule, and can be hypopigmented or hyperpigmented. Generally, the lesions take the form of round or oval macules or papules, although in advanced cases the lesions may become confluent (13,21,33).

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Temperature conditions such as growth at 37°C and production of enzymes, including lipases, phospholipases and proteases are associated with pathogenicity (10,28,39).

The aim of this study was to detect, isolate and identify the occurrence of *Malassezia* species in the skin of clinically healthy students and also in those with macules with a slight fawn discoloration; the isolates were characterized as to the pathogenicity factors as growth at 37°C, lipase, phospholipase and protease activities.

To investigate the distribution of *Malassezia* species in 100 students (50 males, 50 females, aged 10–17 years old) scrapping of skin surface and scalp from 85% clinically healthy and 15% with macules a slight fawn discoloration, were obtained. The students were from a private school Recife – Pernambuco, Brazil. Skin samples were taken from scrapping of five different body sites (abdomen, arms, back, neck and face) and for microscopic examination, were added to samples 10-20% potassium hydroxide to help to dissolve the keratin and debris, facilitating observation of fungal elements. Cultures were obtained on Sabouraud agar medium (Difco) added of olive oil and chloranphenicol (19,32). The isolates were identified by their morphological and physiological properties (catalase, capacity to assimilate tween in different concentrations) according to Duarte (5) and Guillot and Bond (12), and were cultured on Sabouraud agar medium (Difco) added of olive oil on Petri dishes incubated at room temperature (RT = 28°C) and at 37°C and the growth was observed for 10 - 15 days. For detection of lipase activity, it was used the method described by Sierra (34). Cultures were inoculated on Sorbitan monolaurate agar medium and observed for 10 to 15 days for formation of an opaque zone of precipitation.

The detection of phospholipase activity, two methods were used: (29) using Sabouraud agar medium added of 1 M sodium chloride, 0.005 M calcium chloride and 2g egg lecithin; the method of (30) was modified as follows: yeasts were transferred to the center of a Petri dish containing Sabouraud agar medium added of 1 M sodium chloride, 0.005 M calcium chloride and two egg yolks, replacing the “egg yolk” of Difco Laboratories. After 15 days, the colonies were observed and when an opaque halo was present, it was measured in centimeters. This was performed by calculating the activity zone (Pz), represented by the colony diameter, divided by the diameter of the colony plus the zone of precipitation. When the Pz was equal to 1.0, the samples were considered as negatives and when the Pz was less than 1.0, the phospholipase activity was considered as positive.

For protease activity the method proposed by Hankin and Anagnostakis (16) was used. Each isolate was grown on culture medium containing beef extract (3 g), peptone (5 g), gelatin (8%), agar (15 g) and distilled water (1000 mL). The yeast colonies were observed at the 15th day for presence of a halo. The caseine medium, proposed by Lacaz *et al.* (21) and containing: 1th phase - skin milk (10 g) and distilled water (100 mL), 2nd phase - ágar (2

g) and distilled water (100 mL) was also used. At 15th day, when a transparent halo around of the colony was present, acidified solution of mercury chlorate was added to ascertain whether the halo occurred by action of metabolic products acidic or alkaline from the milk, or if there was a true proteolysis indicating that caseine was really degraded. For detection of enzymatic activity, each isolate was growth on the surface of Sabouraud agar medium added of olive oil.

In our study, 114 clinical samples were obtained, being 99 (86.9%) from healthy skin and 15 (13.1%) from skin macules. Direct microscopic examination of the healthy skin revealed globose and spherical yeast cells in 60 (60.6%), yeast cells and hypha in one (1.1%). Negative fungal structures were observed in 38 (38.3%). Of the 15 clinical samples, yeast cells were observed in 12 (80%) skin macules, and both yeast and hypha cells in three (20%). The yeast and hypha phases observed in this study were associated to *Malassezia* pathogenicity (25). *Malassezia* organisms were isolated in six (54.5%) apparently healthy skin and in five (45.5%) skin macules. Nevertheless, *Malassezia* organisms could also be found in normal skin (1,10,21,33) (Table 1).

Malassezia colonies developed in 10 days at 28°C. The colonies were raised and smooth initially and get dry and wrinkled in time. The color of *Malassezia* colonies was white to creamy (18,22,36). The predominant microscopic structures were globose to ellipsoidal and unipolar budding yeasts cells and occur in small collarettes (18,22,36). Seven (64%) isolates were identified as *M. furfur* assimilating well tween 20, 40, 60, 80 and four (36%) *M. simpodialis* assimilated less tween 20 and more tween 40, 60 and 80. According to Guillot and Bond (12) the identification of the species of *Malassezia* by tween assimilation test is used for differentiation among *M. furfur*, *M. simpodialis*, *M. slooffiae*, *M. globosa* and *M. obtusa*. The catalase test of the isolates of *Malassezia* was positive; these results confirmed that might not be *M. restricta* by lack of the enzyme (5,12).

Fungal structures were observed in clinical samples of 47 (65.3%) male and 25 (34.7%) female students. The occurrence of *Malassezia* is verified in both sex (6,7,33) but the prevalence of pityriasis versicolor is observed more frequently in male

Table 1. *Malassezia* spp in apparently healthy and with macules skin.

Sample (n)	Presence of		Negative	<i>Malassezia</i> spp Positive culture (%)
	Y	Y+H		
Healthy skin (99)	60	1	38	6 (6.06)
Macule skin (15)	12	3	0	5 (33.33)
Total	72	4	38	11 (9.64)

Y= Yeast; H= Hypha.

(21,27), however other authors indicated that the incidence is higher in women (31). The role of sex in propensity to development of pityriasis versicolor is still unclear (37).

Students age ranged from 10 to 17 years old, being 19 included 10-11; 35 of 12-13; 17 of 14-15 and one of 16-17 years old. The prevalence of fungal structures in clinical samples was superior in the group of 12-13 years old. Pityriasis versicolor is considered uncommon in children (13) but other authors observed the highest prevalence in 20-30 year-old groups (4,24).

Species of *Malassezia* isolated from healthy skin and with macules varies with the age, sex and body site (8,9,14,21,31). *M. furfur* and *M. sympodialis* grew in both temperature conditions, at RT and 37°C. Similar results are cited by Imwidthaya *et al.* (19) and Lacaz *et al.* (21) who verified that *Malassezia* isolates exhibit better development at 35°-37°C.

Malassezia produces many enzymes including lipases and proteases. All *Malassezia* isolates here studied presented lipase activity including seven (64%) of *M. furfur* and four (36%) of *M. sympodialis*. According to Plotkin *et al.* (28) the characterization of the fungi by lipophilic system can justify the increase of *M. furfur* infections related as changes in skin lipidic layers. No cultures presented phospholipase activity. These findings differ from those obtained by Muhsin *et al.* (26) that detected phospholipase activity in *Malassezia* isolates. Protease activity was verified in four (36.4%) isolates by the formation of a transparent halo around the colony, including two *M. furfur* and two *M. sympodialis*. Mushing *et al.* (26) did not observe protease activity in *Malassezia* isolates, differing from our results.

Probably, these enzymes are able to penetrate in host cells. Electronic microscopy shows enzymatic actions involved during the growth and penetration of the yeast in host cell, confirming the pathogenicity of *Malassezia* isolates (23).

We found no differences as to the pathogenicity factors among species of *Malassezia* isolated from students with healthy skin or with macules.

RESUMO

Identificação e patogenicidade de espécies de *Malassezia* isoladas de pele humana saudável e com mácula

A ocorrência de espécies *Malassezia* em estudantes clinicamente sadios e com máculas com leve descoloração foi investigada e os isolados caracterizados quanto a fatores de patogenicidade como crescimento a 37°C, detecção de lipase, fosfolipase e protease. Amostras clínicas de 100 estudantes de ambos os sexos, diferentes idades e sítios corpóreos foram obtidas por escarificação da superfície da pele e do couro cabeludo e examinadas com hidróxido de potássio e submetidas à cultura. As culturas foram obtidas em meio ágar Sabouraud adicionado

de óleo de oliva, em placa de Petri, incubadas a temperatura ambiente e a 37°C. A identificação foi realizada através das propriedades morfológicas e fisiológicas. A detecção de lipase, fosfolipase e protease foi analisada em meios específicos em placa de Petri pela formação de halo. O exame microscópico direto mostrou células globosas, esféricas e hifa. *Malassezia furfur* foi detectada em sete amostras e *M. sympodialis* em quatro. Todas as culturas apresentaram atividade lipásica, mas nenhuma foi fosfolipase positiva. A atividade proteásica foi observada em dois isolados de *M. furfur* e dois de *M. sympodialis*.

Palavras-chave: *Malassezia furfur*, *M. sympodialis*, patogenicidade

REFERENCES

1. Crespo, E.V.; Delgado, F.V. *Malassezia* species in skin diseases. *Curr. Opin. Infect. Dis.*, 15, 133-142, 2002.
2. Crespo, E.V.; Martos, O.J.; Casaño, A.V. *Malassezia globosa* as the causative agent of pityriasis versicolor. *Br. J. Dermatol.*, 143, 799-803, 2000.
3. Crespo, E.V.; Ojeda M.A.; Vera, C.A.; Crespo, E.A.; Sanches, F.F.; Guého, E. Mycology of pityriasis versicolor. *J. Mycol. Med.*, 9, 143-148, 1999.
4. Crespo, E.V.; Ojeda M.A.; Vera, C.A. Isolation and identification of *Malassezia* spp in pityriasis versicolor, seborrheic dermatitis and healthy skin. *Rev. Iberoam. Micol.*, 16, S16-S21, 1999.
5. Duarte, E.R. Prevalence of *Malassezia* spp. in the ears of asymptomatic cattle and cattle with otitis in Brazil. *Med. Mycol.*, 37, 59-162, 1999.
6. Faergemann, J.; Aly, R.; Maibach, H. Quantitative variations in distribution of *Pityrosporum orbiculare* on clinically normal skin. *Acta Dermatol. Venerol.*, 63, 346-348, 1983.
7. Ferreira, A.W.; Ávila, S.L.M. *Diagnóstico laboratorial das principais doenças infecciosas e auto-imunes*. Guanabara Koogan, Rio de Janeiro, 1ª ed., 1996, 302p.
8. Fisher, F.; Cook, N.B. *Micologia: Fundamentos e diagnósticos*. Reviter, São Paulo, 5ª ed., 2001, 337p.
9. Furtado, M.S.S.; Cortez, A.C.A.; Ferreira, J.A. Pityriase versicolor em Manaus, Amazonas – Brasil. *An Brasil Dermatol.*, 72, 349-351, 1997.
10. Gordon, M.A. Lipophilic yeastlike organisms associated with tinea versicolor. *J. Invest. Dermatol.*, 17, 267-272, 1951.
11. Gueho, E.; Midgley, G.; Guillot, J. The genus *Malassezia* with description of four new species. *Antonie van Leeuwenhoek*, 69, 337-355, 1996.
12. Guillot, J.; Bond, R. *Malassezia pachydermatis*: a review. *Med. Mycol.*, 37, 295-306, 1999.
13. Gupta, A.K.; Bluhm, R.; Summerbell, R. Pityriasis versicolor. *J. Eur. Acad. Dermatol. Venereol.*, 16, 19-33, 2002.
14. Gupta, A.K.; Kohli, Y. Prevalence of *Malassezia* species on various body sites in clinically healthy subjects representing different age groups. *Med. Mycol.* 42, 35-42, 2004.
15. Gupta, A.K.; Kohli, Y.; Summerbell, R.C.; Faergemann, J. Quantitative culture of *Malassezia* species from different body sites of individuals with or without dermatoses. *Med. Mycol.*, 39, 243-251, 2001.
16. Hankin, L.; Anagnostakis, S.L. The use of solid media for detection of enzymes production by fungi. *Mycologia*. 67, 597-607, 1975.
17. Hirai, A.; Kano, R.; Makimura, K.; Duarte, E.R.; Hamdan, J.S.; Lachance, M.A.; Yamaguchi, H.; Hasegawa, A. *Malassezia nana* sp.

- nov., a novel lipid-dependent yeast species isolated from animals. *Int. J. Syst. Evol. Microbiol.*, 54, 623-627, 2004.
18. Hoog, G.S.; Guarro, J.; Gene, J.; Figueras, M.F. *Atlas of Clinical Fungi*. Centraalbureau voor Schimmelcultures, Universitat Rovira i Virgili, Spain, 1 ed., 1995, 720p.
 19. Imwidththaya, P.; Thianprasit, M.; Smiruang, S. A study of pityriasis versicolor in Bangkok (Thailand). *Mycopathology.*, 105, 157-161, 1989.
 20. Juncosa Morros, T.; Gonzalez-Cuevas, A.; Alayeto Ortega, J. Cutaneous colonization by *Malassezia* spp in neonatos. *An. Esp. Pediatr.*, 57, 452-456, 2002.
 21. Lacaz, C.S.; Porto, E.; Martins, J.E.C.M.; Heins-Vaccari, E.M.; Melo, N.T. *Tratado de Micologia Médica*. SAVIER, São Paulo, 9 ed., 2002, 1104p.
 22. Larone, D.H. *Medically Important Fungi - A Guide to Identification*. ASM, Washington, 3^a ed., 1995, 274p.
 23. Mayser, P.; Laabs, S.; Hever, K.; Gruder, K. Detection of extracellular phospholipase activity in *Candida albicans* and *Rhodotorula rubra*. *Mycopathology.*, 135, 149-155, 1996.
 24. Midgley, G.; Gueho, E.; Guillot, J. Disease caused by *Malassezia* species. In: Ajello, L.; Hay, R.J. *Topley and Wilson's Microbiology and microbial infections*. Arnold, London, 1998, p.201-211.
 25. Moreno-Gimenez, J.C. Pityriasis versicolor y otros procesos relacionados. *Monografías de Dermatología*, 3, 233-243, 1995.
 26. Muhsin, T.M.; Aubaid, A.H.; Al-Duboon, A.H. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. *Mycoses*, 40, 465-469, 1997.
 27. Nakabayashi, A.; Sei, Y.; Guillot, J. Identification of *Malassezia* species isolated from patients with seborrheic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. *Med. Mycol.*, 38, 337-341, 2000.
 28. Plotkin, L.I.; Squiquera, L.; Mathov T.; Galimberti R.; Leoni, J. Characterization of the lipase activity of *Malassezia furfur*. *J. Med. Veter. Mycol.*, 34, 43-48, 1996.
 29. Price, M.F.; Cawson, R.A. Phospholipase activity in *Candida albicans*. *Saboraudia*, 15, 79-185, 1977.
 30. Price, M.F.; Wilkinson, I.D.; Gentry, L.O. Plate method for detection of phospholipase activity in *Candida albicans*. *Saboraudia*, 20, 7-14, 1982.
 31. Prohic, A. Identification of *Malassezia* species isolated from scalp skin of patients with psoriasis and health subjects. *Acta Dermatoven. Croat.*, 11, 10-16, 2003.
 32. Rippon, J.W. *Medical Mycology: The pathogenic fungi and the pathogenic actinomycetes*. W. B. SAUNDERS COMPANY, United States of America, 2^a ed., 1982, 842p.
 33. Sidrim, J.J.C.; Rocha, M.F.G. *Micologia Médica à Luz de Autores Contemporâneos*. Guanabara koogan, Rio de Janeiro, 1^a ed., 2004, 388p.
 34. Sierra, G. A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie Van Leeuwenhoek Ned. Tijdschr. Hyg.*, 23, 15-22, 1957.
 35. Sugita, T.; Tajima, M.; Takashima, M.; Amaya, M.; Saito, M.; Tsuboi, R.; Nishikawa, A. A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. *Microbiol. Immunol.*, 48, 579-583, 2004.
 36. Sutton, D.A.; Fothergill, A.W.; Rinaldi, M.G. *Guide to Clinically Significant Fungi*. Williams & Wilkins, Baltimore, MD, 1^a ed., 1998, 180p.
 37. Tarazooie, B.; Kordbacheh, P.; Zaini, F.; Zomorodian, K.; Saadat, F.; Zeraati, H.; Hallaji, Z.; Rezaie, S. Study of the distribution of *Malassezia* species in patients pityriasis versicolor and healthy individuals in Tehran, Ira. *BMC Dermatol.*, 4, 1-46, 2004.
 38. Velegraki, A.; Alexopoulos, E.C.; Kritikous, S.; Gaitanis, G. Use of fatty acid RPMI 1640 media for testing susceptibilities of eight *Malassezia* species to the new triazole posaconazole and to six established antifungal agents by a modified NCCLS M27-A2 microdilution method and etest. *J. Clin. Microbiol.*, 42, 3589-3593, 2004.
 39. Vidotto, V.; Leone, R.; Sinicco, A.; Itokuwa S.; Criseo, G. Comparison of phospholipase production in *Cryptococcus neoformans* isolates from AIDS patients and bird droppings. *Mycopathology*, 14, 71-76, 1998.0