

The Lipase Activities of *Malassezia* Species Isolated from Seborrhoeic Dermatitis/Dandruff Patients

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ABSTRACT

Introduction: *Malassezia* a commensal yeast, can become pathogenic leading to different clinical forms of dermatosis. Being an obligatory lipophilic, the lipolytic enzymes produced by them help in their growth by obtaining useful lipids from the environment further contributing to the pathogenesis of dermatosis. There are no comparative reports concerning secreted lipase activities of *Malassezia* species associated with Seborrhoeic Dermatitis/Dandruff (SD/D).

Aim: To analyse and compare lipase activities of *Malassezia* spp. isolated from SD/D patient's lesional area and Healthy Controls (HC) and correlates this feature in pathogenesis of SD/D.

Materials and Methods: Lipase activities of *Malassezia* species isolated from lesional area of SD/D patients and HC were analysed and compared. Extracellular proteins of *Malassezia* spp. were extracted from the Leeming and Notman agar after removing and weighing the yeast cells from the agar surface.

The lipase activity was measured using an assay based on hydrolysis of p-Nitrophenylpalmitate (pNPP). ANOVA test and GraphPad were used for statistical analysis.

Results: *Malassezia furfur* wet fungal weight was higher (1.1±0.13 g) compared to *M. globosa* (0.36±0.4 g) and *M. restricta* (0.26±0.4 g). The mean extracellular lipase activity of *M. globosa* (0.15 U) and *M. restricta* (0.18 U) was significantly higher than *M. furfur* (0.08 U) isolates (p<0.001). No significant difference was observed among *M. globosa* (SD/D-0.15 U vs. HC-0.14 U), *M. restricta* (SD/D-0.2 U vs. HC-0.16 U) and *M. furfur* (SD/D-0.08 U vs. HC-0.1 U) isolated from SD/D patient's lesional area and HC.

Conclusion: The lipase activity of *M. globosa* and *M. restricta* was higher despite slow growth rate. Lipase activity alone may not play a role in the onset of SD/D. As the pathogenic mechanism of fungi is complex, it is difficult to explain the pathogenicity of *Malassezia* based only on lipase activities.

Keywords: Lipase activity, P-nitrophenylpalmitate, Seborrhoeic dermatitis

INTRODUCTION

Members of the genus *Malassezia* are commensal yeasts that reside on the skin of human and many warm-blooded animals. Under the influence of diverse environmental factors such as temperature and humidity or immunodeficiency it can become pathogenic leading to different clinical forms of dermatosis like pityriasis versicolor, seborrhoeic dermatitis/dandruff (SD/D), *Malassezia* folliculitis and some subsets of psoriasis and atopic dermatitis [1]. *Malassezia* can also cause serious infections such as catheter associated sepsis [2, 3]. There are totally 15 *Malassezia* spp. described till date based on molecular characterisation [4]. Lipolytic enzymes secreted by them could help them in utilising the lipids from the immediate environment further contributing to the pathogenesis of dermatoses. Lipolytic enzymes such as lipase, esterase, phospholipase, and lysophospholipase are considered to be closely associated with the virulence of *Malassezia* [5]. However, due to fastidious nature of *Malassezia* (especially *M. globosa* and *M. restricta*), research on analysis of these virulence factors are restrained.

The SD/D is a common skin disorders associated with *Malassezia*, occurring in at least 40-50% of the general population of immunocompetent adults [6,7]. Analysis of the complete genome of *M. globosa* and the partial genome of *M. restricta* have presented gene-encoding enzymes of the lipase and phospholipase families that could explain the lipid dependency of the genus [8]. It seems likely that these fungi would also require lipid for growth on human skin and that lipase-mediated digestion of sebum glycerides would be the first step in lipid consumption. There are no comparative reports concerning the secreted lipase activities of *Malassezia* species associated with SD/D. Hence, the aim of the present study was to analyse and compare lipase activities of *Malassezia* species

isolated from lesional area of SD/D patients and HC and correlate this feature in pathogenesis of SD/D.

MATERIALS AND METHODS

The prospective case-control study was conducted in villages of northwestern India (Punjab) and northern India (Haryana) during December 2011 to February 2013. The present study was conducted at the departments of Medical Microbiology and Dermatology, Venereology and Leprosy, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Study population was categorised as SD/D patients or healthy individuals as per the previous conducted study [4]. Inclusion criteria were based on the presence of inflammation of the scalp in the form of red, scaly and flaking rash. Cases applying topical antifungals and/or steroid to the scalp preceding a month of sampling were excluded from the study. Specimen was collected using a blunt scalpel from relatively severely affected areas and vertex region of SD/D patients and healthy individuals scalp respectively. The specimen collection was carried out at convenience by random screening. The degree of SD/D severity was graded as mild, moderate and severe based on size, colour and surface of the flakes/scales [9]. All isolates were identified based on phenotypic and molecular technique as described earlier [10]. Total 30 clinical isolates of *Malassezia* isolated from SD/D patient's lesional area and from HC were randomly selected and included in the study. Isolates include *M. globosa*, n=10 (5 SD/D, 5 HC); *M. restricta*, n=10 (5 SD/D, 5 HC) and *M. furfur*, n=10 (5 SD/D, 5 HC). The study protocol was approved by the Institute Ethics Committee of Postgraduate Institute of Medical Education and Research, Chandigarh, India. The samples were collected after obtaining informed consent of

the subjects. All strains were grown on Leeming and Notman Agar (LNA) media and incubated at 34°C for seven days.

Each *Malassezia* isolate (20 CFU/mL) was cultured on five sets of LNA plates under the condition described above. *Malassezia* cells were scrapped from the surface of LNA plate and transferred to preweighed eppendorf tube. The wet weight of *Malassezia* cells was noted. After scraping the *Malassezia* cells from the surface of agar medium, the surface was rinsed twice with distilled water. LNA medium was crushed in pestle-mortar. The resultant slurry was incubated in 100 mL protein extraction buffer at 4°C for 24 hour. The mixture was filtrated through Whatman filter paper followed by 0.2 mm membrane filter (Millipore Corporation, MA, USA). The resultant solution was concentrated using Amicon Ultra centrifugal filter, ultracel- 30K (Millipore corporation, MA, USA) [5].

Lipase activity was measured using an assay based on hydrolysis of p-Nitrophenylpalmitate (pNPP). Due to low solubility in water, pNPP (Sigma, MO, USA) was first dissolved in 2-propanol at 10 mM concentration then 1 mM pNPP was dissolved in 10 mM phosphate buffer (pH=6) and 1% TritonX-100. To this substrate, 30 µL of concentrated extracted extracellular protein was added and incubated at 37°C for one hour. Before measuring the absorbance at 405 nm, 200 mL of 1 M Tris buffer (pH=8.0) was added to stabilise the pH-dependent dye pNP (p-Nitrophenol). Release of pNP from pNPP was measured in an ELISA reader as the increase in absorption at 405 nm [11].

STATISTICAL ANALYSIS

Comparison of lipase activity among *M. globosa*, *M. restricta* and *M. furfur* was analysed by ANOVA (Tukey's multiple comparisons) test and calculated using the GraphPad Prism version 6.01 (www.graphpad.com). p<0.05 was considered as statistically significant.

RESULTS

After seven days of incubation on LNA media, *M. furfur* average wet fungal weight was higher (1.1±0.13 g), compared to *M. globosa* (0.36±0.4 g) and *M. restricta* (0.26±0.4 g) [Table/Fig-1]. The protein concentration was normalised, as the wet weight of *M. furfur* was ~3 and ~2 times higher than *M. globosa* and *M. restricta*. One unit of lipase activity is defined as the amount of enzyme that released 1 µM of p-nitrophenol per minute [11]. The mean extracellular lipase activity of *M. globosa* (0.15 U) and *M. restricta* (0.18 U) was significantly higher than *M. furfur* (0.08 U) isolates (p<0.0001) [Table/Fig-2,3]. No significant difference in the extracellular lipase activity was observed among *M. globosa* (SD/D-0.15 U vs. HC-0.14 U; p=0.5818), *M. restricta* (SD/D-0.2 U vs. HC-0.16 U; p=0.0671) and *M. furfur* (SD/D-0.08 U vs. HC-0.1 U; p=0.4067) isolated from SD/D patient's lesional area and HC [Table/Fig-4]. As there was no statistical difference between isolates from SD/D patients and HC, degree of SD/D and lipase activity was not calculated.

<i>M. globosa</i>				<i>M. restricta</i>				<i>M. furfur</i>			
SD/D patients	Weight in gm	Healthy controls	Weight in gm	SD/D patients	Weight in gm	Healthy controls	Weight in gm	SD/D patients	Weight in gm	Healthy controls	Weight in gm
U10	0.44	NU11	0.38	U54	0.23	NU55	0.32	U5	1.12	NU5	1.25
U66	0.36	NU68	0.38	U64	0.28	NU63	0.29	U60	1.14	NU9	1.35
U71	0.38	NU70	0.43	U65	0.28	NU65	0.32	U62	1.32	NU33	1.2
U77	0.37	NU75	0.45	U73	0.25	NU78	0.3	U82	1.34	NU35	1.28
U60	0.39	NU60	0.47	U74	0.30	NU80	0.27	U89	1.3	NU62	1.29

[Table/Fig-1]: Isolation source and wet weight (in grams) of different *Malassezia* isolates.

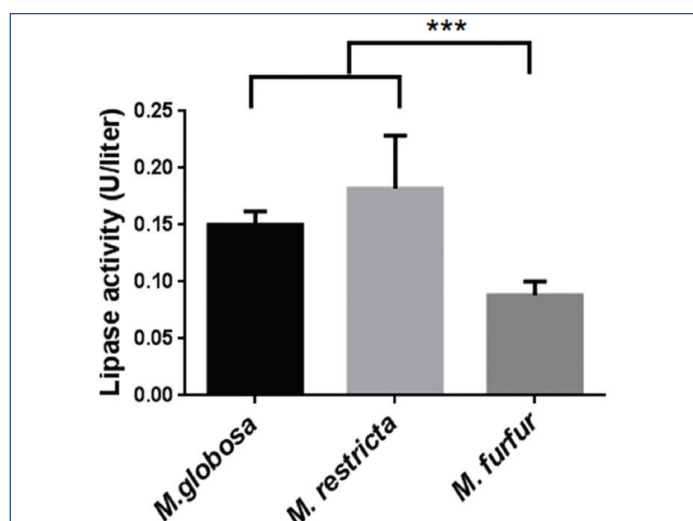
Abbreviation: SD/D, Seborrhoeic dermatitis/dandruff

U and NU are just the lab number in which U indicates SD/D patient isolate and NU indicates HC isolates

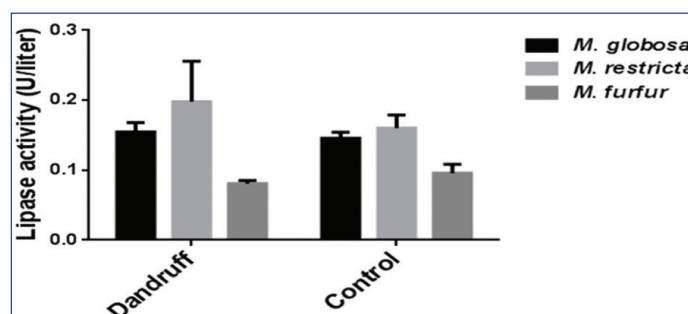
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	p-value
<i>M. globosa</i> vs. <i>M. restricta</i>	-0.03187	0.06466 to 0.0009227	ns
<i>M. globosa</i> vs. <i>M. furfur</i>	0.06181	0.03025 to 0.09337	<0.001
<i>M. restricta</i> vs. <i>M. furfur</i>	0.09368	0.05924 to 0.1281	<0.0001

[Table/Fig-2]: Statistical analysis of the mean extracellular lipase activity of *M. globosa*, *M. restricta* and *M. furfur* isolates.

Test employed: ANOVA (Tukey's multiple comparisons) test. p<0.05 was considered as statistically significant



[Table/Fig-3]: Lipase activity in extracted extracellular proteins of different *Malassezia* species. Error bars indicate the standard deviations of the background-subtracted values for three independent replicate experiments (* p<0.05- significant).



[Table/Fig-4]: Lipase activity in extracted extracellular proteins of *Malassezia* species isolated from seborrhoeic dermatitis/dandruff patient's lesional area and healthy controls.

Error bars indicate the standard deviations of the background-subtracted values for three independent replicate experiments.

DISCUSSION

The pathobiology of *Malassezia* in SD/D is not clear, due to the complex interaction of the commensal yeast with the skin. *Malassezia* shows lipid dependency due to the absence of fatty acid synthase gene. *Malassezia* yeast secretes the extracellular lipolytic enzymes to obtain saturated fatty acids from the environment [8]. The lipolytic enzymes (lipase, phospholipase, esterase and lysophospholipase) may be associated with the virulence of *Malassezia* in SD/D.

Juntachai W et al., compared the extracellular lipase activity of standard strains of seven *Malassezia* spp. *M. globosa* (CBS 7986) had the highest lipase activity compared to the other six *Malassezia* spp. (*M. pachydermatis*, *M. restricta*, *M. furfur*, *M. slooffiae*, *M. sympodialis* and *M. obtusa*) [5]. However, there are no relative reports regarding secreted lipase activities of *Malassezia* species associated

with SD/D. In the present study the lipase activity of *M. globosa* and *M. restricta* was higher despite slow growth rate (the wet weight of species indicate the growth rate; higher the wet weight higher the growth rate) contrasting the results of previous study [5]. This difference may be due to source of isolation. In the previous study the standard strains were used to compare the lipase activity, whereas in the present study the isolates were from SD/D patients and HC [5]. This is the first in-vitro study to investigate the lipase activity between the *Malassezia* strains isolated from SD/D patient's lesional area and healthy controls. Recently Park M et al., characterised *MrLip1*, lipase gene of *M. restricta*, isolated from a SD patient. However, it would have been interesting to characterise expression of this gene in *M. restricta* isolated from healthy individual [12].

Ran Y et al., for the first time studied the role of lipase and its significance in *M. furfur*. The lipase was detected in the insoluble fraction of the cells and was acidic in nature. Association between cell growth and lipase activity was observed [13]. Brunke S et al., in 2006 cloned and characterised *MfLIP1* (gene encoding extracellular lipase of *M. furfur*) [11]. Conserved lipase motif of *C. albicans* has shown to be similar to *MfLIP1*. In the same year, Shibata N et al., cloned and characterised *M. pachydermatis* extracellular lipase gene [14]. But both *M. furfur* and *M. pachydermatis* have least association in the causation of SD/D. Many reports including the previous study have shown that *M. globosa* and *M. restricta* are the species most commonly associated with SD/D [9]. Even the whole genome sequence of *M. globosa* has revealed the presence of 14 lipase-encoding genes, of which 13 were predicted secreted proteins [8]. However, only two genes (*MgLIP1* and *MgLIP2*) have been isolated and characterised [15,16]. It also contained conserved lipase motif. Late log phase of *M. globosa* expressed higher *MgLIP1*. Due to fastidious nature of *M. restricta*, its lipase activity was not detected. In addition, recently *MgLIP1* expression was detected on the human scalp [17].

In the present study, in a given species no difference has been observed in the lipase activity among the strains isolated from lesional area of SD/D patients and HC. These results suggest that lipase activity alone may not play a role in the onset of SD/D. However, lipase may help in metabolising lipids and integrating the products on cell wall leading to growth and survival in the environment. As the pathogenic mechanism of fungi is complex, it is difficult to explain the pathogenicity of *Malassezia* based on lipase activity alone. Other virulence factors of *Malassezia* species may play a role in pathogenesis either alone or in combinations. The activity of phospholipase is an important virulence that has been detected in *M. pachydermatis* and implicated in the occurrence of skin lesions [18]. In addition, secreted phospholipase activities of *M. furfur* were detected in vitro by the egg-yolk method [19]. Quantitative analysis and comparison of phospholipase activities of *M. globosa* and *M. restricta* isolated from SD/D patient's lesional area and HC may provide more insight on the pathogenesis of SD/D.

LIMITATION

The limitation of present study was the low number of *Malassezia* strains studied, due to difficulty in cultivating these fastidious species.

Due to small number of strains we couldn't comment on the degree/severity of SD/D and lipase activity. Storage of the *Malassezia* strains for longer period may have reduced the enzyme activity.

CONCLUSION

Despite slow growth rate of *M. globosa* and *M. restricta*, the lipase activity was higher than *M. furfur*. No difference was observed in the lipase activity among the strains isolated from lesional area of SD/D patients and HC. *Malassezia* pathogenesis is a complex mechanism. Hence, lipase activity alone may not play a role in the causation of SD/D.

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