Research Article

Detection of phospholipase production by EGG Yolk-Agar in *Malassezia* isolates from diseased and healthy human host

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ABSTRACT

Background: *Malassezia* species (spp.) are cutaneous opportunistic pathogens and associated with various dermatological diseases. Virulence traits include a variety of enzymes such as lipases, phospholipase, and hyaluronidase. Phenotypic determination of phospholipase production in isolates from diseased group can confirm the pathogenic potential of the enzyme. **Objective:** To find out the presence of phospholipases activity in the isolates of *Malassezia* species from three dermatologic conditions, i.e., atopic dermatitis, seborrheic dermatitis, and pityriasis versicolor; and comparative analysis with isolates from healthy host. **Materials and Methods:** It was a prospective observational study conducted for a period 12-month to determination and quantify extracellular phospholipase activity (Pz) by the EGG Yolk-Agar method. **Results:** Determination of extracellular Pz, by the EGG Yolk-Agar method was done on all the *Malassezia* isolates, for assessing its role as a potential virulence factor. Statistically significant results, for the phospholipase, were obtained in the patient group for *Malassezia sympodialis*. **Conclusions:** The study demonstrated an association between pathogenic isolates and phospholipase production as compared to isolates from the healthy host. Such findings highlight the importance of phospholipase enzyme in virulence determination of a yeasts which is generally considered a commensal.

KEY WORDS: *Malassezia sympodialis*; Phospholipase; Extracellular; Dermatitis; Pityriasis Versicolor

INTRODUCTION

Basidiomycete yeasts of the genus *Malassezia* are lipophilic, unipolar, and budding yeasts characterized by a thick cell wall with high lipid content, an incomplete fatty acid synthesis metabolic pathway, and dependence on a variety of extracellular enzymes such as lipases, phospholipases, and acid sphingomyelinases for survival.[1] An exception to this is *Malassezia pachydermatis* which is able to survive in axenic culture without external lipid source.[1-3]

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In recent years, *Malassezia* has garnered a lot of interest in both clinical and microbiological society. Investigations of the skin microbiome using molecular methods have discovered the abundance of *Malassezia* among eukaryotes on all human surface body sites. Some studies have suggested their presence in sites beyond the skin, including the human oral microbiome.[4]

Malassezia are associated with a variety of disorders such as seborrheic dermatitis (SD), dandruff and atopic dermatitis (AD), folliculitis, and systemic diseases.[5,6]

The pathogenic potential of *Malassezia*, a known commensal is related to virulence factors, environmental factors, and genetic predisposition of host.[5,6]

Virulence factors of various pathogenic microbes include secretory hydrolytic enzymes (proteinases and the lipolytic

enzymes, lipases, and phospholipases) that help in entry into host tissue by destabilization of the host cell membrane leading to release of lipid second messengers.[7,8] Role of proteinases has been well established in the pathogenicity of microbes and induction of inflammatory response. In contrast, the roles of lipases and phospholipases in virulence remain widely unexplored. Lipases catalyze the hydrolysis of ester bonds of triacylglycerol whereas Phospholipases hydrolyze one or more ester linkages in glycerophospholipids, resulting in the release of free fatty acids which are highly reactive and induce pain and inflammation.[9] Virulence potential of lipases is known for the pathogens such as *Propionibacterium acnes* and *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa, Mycobacterium tuberculosis,* and *Candida albicans* the expression of which is largely influenced by the stage of infection.[7,8]

Another pathogenic and commensal fungus prevalent of human skin is *C. albicans*, which though distantly related have similar gene families encoding for enzymes phospholipases, lipases, aspartyl proteases, and acid sphingomyelinases.[7]

The role of phospholipases in host cell penetration, injury, and lysis has been documented in the study of parasitic protozoa such as *Toxoplasma gondii* and *Entamoeba histolytica*, *Rickettsia* spp. and *S. aureus*, and fungal pathogens *C. albicans*, and *Cryptococcus neoformans*.[8]

Current information on phospholipases in *Malassezia* spp. is limited.[10,11] Extracellular phospholipase activities (Pzs) in *Malassezia furfur*, *M. pachydermatis*, *Malassezia slooffiae*, *Malassezia sympodialis*, *Malassezia globosa*, *Malassezia restricta*, and *Malassezia obtuse* have been studied.[11] However, only *M. pachydermatis* isolated normally dogs demonstrated significant Pz. Very few studies on Pz in *Malassezia* spp. associated with humans have been attempted which shows relatively low level of activity implying marginal role of these enzymes in human disease.[1,10-12]

The purpose of the study was to find out the presence of Pz in the isolates of *Malassezia* species from three dermatologic conditions, i.e., AD, SD, and pityriasis versicolor (PV); and comparative analysis with isolates from healthy host.

MATERIALS AND METHODS

The study was conducted for the period of 12-month (January 2012-January 2013) including enrolment, analysis, and compilation of data.

It was a prospective observational study conducted in the Department of Microbiology and Department of Dermatology and Venereology in University College of Medical Sciences & GTB Hospital, Delhi.

A total of 102 isolates were obtained from both patients (AD, SD, and PV) and healthy controls. Speciation of the *Malassezia* isolates was done using the phenotypic method, which included morphological characteristics (macroscopic morphology and microscopic morphology), and physiological methods (urease test, catalase test, β-glucosidase test, tween assimilation test, and growth at 37°C and 40°C).[13,14]

Stock cultures were maintained at −20°C in sterile for further use.

Determination and quantification of extracellular Pz by the EGG Yolk-Agar method.[15,16]

Yeasts were sub cultured on mDA at 25°C to ensure purity and viability. Inoculum was prepared by picking five distinct colonies from a fresh culture of *Malassezia* spp. Colonies were suspended in 5 ml of sterile normal saline. The turbidity of the resulting suspension was adjusted visually by adding sufficient sterile saline or more colonies to adjust the turbidity to that produced by a 0.5 McFarland standard. The procedure yielded a yeast stock suspension of 1×10^5 to 5×10^5 yeasts cell/ml. 10 µl aliquot of yeast suspension was spot-inoculated onto the surface of the EGG Yolk-medium and the plates were left to dry at room temperature. Pz was determined by the ratio of the diameter of the colony to total the colony plus the precipitation zone and scored as follows:

- Pz ≥ 0.64 to <1 high Pz
- Pz <0.64 very high activity present
- Pz = 1 null or no activity.

Hence, the higher the phospholipase value, the lower the production of phospholipases. Each strain was tested in duplicate, and the phospholipase recorded as an average of the two phospholipase values for each isolate. Pz was expressed as a mean of phospholipase values.

RESULTS

A total of 76 out of the 102 *Malassezia* culture positive (Table 1) samples grew on EGG Yolk-medium. No isolate of *M. restricta* was able to grow on repeated culture. Thus, evaluation of Pz cannot be done for that species. Determination of Pz on two different occasion was analyzed (Table 2).

Statistically significant differences were found in between the Pz values of day 15 and day 20 subsequent to incubation for *M. sympodialis* strains isolated from diseased group ($P = 0.0012$).

Table 1: Number, species, and origin of strains examined for phospholipase production

Species	*Malassezia* strains				
	PV	AD	SD	Healthy	Total
Malassezia globosa	19	13	18	15	65
Malassezia sympodialis	11	6	5	4	26
Malassezia furfur	2	1	1	1	5
Malassezia restricta	2	4	0	0	6
Total	34	24	24	20	102

PV: Pityriasis versicolor, AD: Atopic dermatitis, SD: Seborrheic dermatitis

Table 2: Phospholipase activity of the *Malassezia* species of all the study group expressed as a Pz value mean after 15 and 20 days of culture

Species	Strain grown on EGG Yolk media	Strain with positive phospholipase	Pz 15 days	Pz 20 days
Malassezia globosa	50	2	1	0.925
Malassezia sympodialis	22	10	0.94	0.85
Malassezia furfur	4	3	0.95	0.80

Pz: Phospholipase activity. Pz: Ratio of colony diameter to total diameter of the colonies plus the precipitation zones

DISCUSSION

In this study, 102 isolates of different *Malassezia* species from different dermatologic condition and healthy controls were obtained. Out of these 74.5% isolates were able to grow on EGG Yolk-Agar for determination of Pz. None of the isolates from healthy skin site elaborated phospholipase in our study. This is a strong indicator suggesting the pathogenic potential of phospholipase enzyme in lesion development.

The method used in this study provided good results for *M. sympodialis* and *M. furfur*, while strains *M. globosa* and *M. restricta*, had limited growth in the chosen medium. Similar findings were observed in a study on human isolates by Pini and Faggi,[17] where *M. obtusa, M. slooffiae, M. globosa,* and *M. restricta* had difficulty developing in EGG Yolk-Agar media.[17] To measure the Pz of *Malassezia*, 8-10 days at least of incubation at 32°C were needed. This is longer in comparison with Pz in *Candida*.[18] Furthermore statistically significant differences were observed on increasing the duration of incubation suggesting the *in vivo* aspect where there is a delayed expression of enzymatic activity. In our study, even though *M. globosa* was the predominant isolate but the Pz was more in *M. sympodialis* and *M. furfur*. 75% of *M. furfur* isolates which were able to grow on EGG Yolk-Agar demonstrated phospholipase in comparison to 45% *M. sympodialis* and 4% *M. globosa*. High Pz in *M. sympodialis* isolates of PV were observed in a similar study.[17] In this study, only one species was isolate per site. Other molecular studies have identified the presence of more than one species from the same site of infection. Metagenomics studies data show that the high abundance of *M. obtusa* with *M. sloofiae* suggesting the possibility that one species could break down the lipids for utilization by both thus ensuring survival of both.[4,6,12]

Our study lacked any *M. pachydermatis* isolate, but a study in Tokyo used assay based on hydrolysis of L-a-phosphatidylcholine dimyristoyl and final colorimetric analysis demonstrated that secreted Pz of lipid-dependent *Malassezia* species were not as high as those of a lipid-independent species, *M. pachydermatis*.[10] This finding is in stark contrast to this study where lipid-dependent isolates showed significant Pz. In various canine studies, Pz was recorded in *M. pachydermatis* cultured from skin lesions which support the proposal of an association between phospholipase production and pathological effect like biofilm production.[15,19-22] Other studies also report the Pz only with relation to skin diseases in *M. pachydermatis*,[19,23] and *M. furfur*.[24] In a study on catheter-associated isolates of *M. furfur* very high levels of phospholipase production was observed as compared to isolates from blood.[25] Vlacho et al. study showing a modification of phospholipase production after exposure to β-endorphin in isolate from lesional skin in human isolates further highlights the relevance of enzyme elaborating strains present in some but not all humans.[26]

Phospholipase gene family is the most expanded family in *Malassezia,* and high expression of such genes can explain optimal survival on host, characteristic symptoms of the disease and niche-specificity such as scalp, chest, and back.[4,26]

Evidence from studies of phospholipases from bacteria associate other functions that facilitate virulence, e.g., signal transduction, stimulation of host cell to release cytokines, and the host inflammatory response.[27-29]

The strength of our study is the phenotypic identification of such virulence activities on human isolates. Very few studies are in literature are there involving so many isolates from a variety of skin diseases.[17,24,30] Phenotypic method of our study gives enough evidence of pathogenic value of phospholipase as none of isolates from healthy controls showed any activity.

Limitation of our study was the use of EGG Yolk-Agar media which is not specific because the EGG Yolk contains substrate for both the phospholipases (phospholipids) and lipases (triglycerides).[27] Also many isolates were unable to grow on this media.

CONCLUSION

In conclusion, this study correlates disease manifestation with isolates elaborating enzymes as compared to isolates from healthy host. Difficulty in isolating the species and also the absence of a good laboratory method to demonstrate enzymatic activity is a hurdle to increase our understanding of these virulence factors. Phospholipase should be considered as one of the many factors involved in the complex interaction between the yeasts and its host leading to the development of the lesions in the skin. Therefore, more studies with larger sample size are needed to understand the relationship between different species with an emphasis on their distribution and role in human diseases. The knowledge of the genetic basis as well as the phenotypic evaluation could help in understanding the mechanism of adaptation to mammal's skin and thus the disease pathogenesis. This open up a vast area of research for the development of newer drugs for the recurrent chronic disease caused by such commensals.

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