

Original article

Detection of biofilm formation in clinical isolates of *malasseziafurfur* isolated from suspected patients of tinea versicolor in a tertiary care hospital

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ABSTRACT

INTRODUCTION: *Malassezia* are lipophilic basidiomycetous yeasts. They are inhabitants of normal skin. They are responsible for a variety of superficial as well as systemic infections of which *Tinea versicolor* is the most common presenting clinical condition.

METHODS: A study was undertaken with 80 clinical samples collected from clinically diagnosed patients of *Tinea versicolor* attending the Dermatology and Venereology outpatient department and the study was carried out in the Department of Microbiology, of Dr B R Ambedkar Medical College and Hospital, Bangalore during a period of 6 months from January 2016 to June 2016. Skin samples were processed by a preliminary direct microscopy-10% KOH wet mount followed by culturing the same on to antibiotic incorporated Sabouraud dextrose agar (SDA) media with antibiotics with an overlay of sterile olive oil and incubated at 37°C and the culture growth observed. Biochemical reactions were done to confirm isolates of *Malassezia furfur* and crystal violet staining was done of the culture to demonstrate biofilm.

RESULTS: The majority of patients were males in the age group of 20-40 years (35%) followed by females of the same age group (30%). 90% of the samples were positive for KOH mount and culture. 7.5% of the samples were negative by KOH mount but positive by culture. 2.5% samples were negative for KOH mount and culture. Out of 80 samples 24 isolates (30%) were confirmed as *Malasseziafurfur*. Positive staining with crystal violet was seen in all isolates of *Malassezia furfur* showing that they produce biofilms.

CONCLUSION: The clinical isolates confirmed to be *Malasseziafurfur* showed the presence of biofilm formation which may be a factor adding to the pathogenicity of *Tinea versicolor* and hypothesizing it as a cause for chronicity following treatment failure.

KEY WORDS: *Malasseziafurfur*, *Tinea versicolor*, Biofilm, Crystal violet

INTRODUCTION

The most common cutaneous yeast infection is *Tinea versicolor*. It is caused by *Malassezia* species which is a lipophilic yeast, which are part of the normal skin flora. They sometimes assume a pathogenic state within the epidermis. When this occurs, patients will present with hypopigmented and/or hyperpigmented scaling lesions on the upper trunk independent of the immune status of the patients [1, 2]. KOH mount is the preliminary, convenient and inexpensive test for diagnosis of superficial fungal infections. Further culture of the sample confirms the diagnosis. Biochemical reactions help in speciation of the isolates. Patients with a propensity for developing *Tinea versicolor* may have a genetic component that predisposes them to disease, either immunosuppression or hyperhidrosis [1, 2]. The other contributing factor is colonization of *Malassezia* species in anatomically vulnerable areas of the skin. Exogenous factors such as sunlight, corticosteroids, and oil-based products encourage the growth of *Malassezia* yeast forms. In response to the mentioned factors, *Malassezia* enters a pathogenic state and clinically manifests as *Tinea versicolor*. Hypopigmentation results from the secondary effect of dopa-tyrosinase inhibition by azelaic acid that is produced by lipases present in *Malassezia* [3]. The phospholipases permit adhesion to the skin which is

the first step in infection. The amount of lipases released by the fungi depends on various factors ranging from pH of the skin to specific receptors present on the cell membrane which is directly proportional to phospholipase levels [1].

For most dermatophytes, a Th1-predominant immune response is evoked but this can be downregulated by metabolites produced by *Malassezia*. There is localization of the organisms to the stratum corneum where immune detection can be bypassed. They generally, do not trigger the innate immune system and thus do not initiate any pathological inflammation. This explains the lack of pruritus and inflammatory response in most patients [3].

Biofilms are composed of a matrix of extracellular polysaccharides, DNA, amyloid and adhesive fibers that causes the permanent adhesion of the colony to biologic and non-biologic surfaces including healthy skin [4]. Fungal biofilms contain yeast and hyphal forms in addition to the above which may contribute to adherence. The biofilm plays an important role in protecting the colony from destruction by antimicrobial agents by either preventing their diffusion or inactivation by direct binding, low pH, and high concentration of metallic ions [4, 5, 6]. It has been suggested that biofilms may be a cause of relapse in non-infectious diseases like Tinea versicolor. Non dividing persisting cells within the biofilms may be responsible for treatment failure as they are immune to most antimicrobial agents by deactivating their apoptosis pathway [4, 7]. Biofilms contribute to resistance to antifungal therapy, increasing the cost and duration of treatment. Most of the times they go unidentified in routine culture. Infection may become chronic despite repeated treatment probably due to biofilm formation [2]. Herein, we can hypothesize that biofilm formation by *Malassezia furfur* can be a potential mechanism behind its transformation from normal skin flora to pathogen and subsequent expression as Tinea versicolor, further leading to chronic Tinea versicolor.

AIMS AND OBJECTIVES:

AIMS:

- To isolate *Malassezia furfur* from clinically diagnosed cases of Tinea versicolor.
- To detect biofilm formation by crystal violet staining of confirmed isolates of *Malassezia furfur*.

OBJECTIVES:

- To hypothesize the role of biofilm formation in the pathogenicity of Tinea versicolor in spite of *Malassezia furfur* being a normal flora of skin and its role adding to chronicity despite treatment with antifungal drugs.

INCLUSION CRITERIA:

- Clinically diagnosed cases of Tinea versicolor.
- Cases of all age groups and both sexes.

EXCLUSION CRITERIA:

- Clinical diagnosis of Tinea nigra and Dermatophytosis.

METHODOLOGY

A study was undertaken with 80 clinical samples collected from clinically diagnosed patients of Tinea versicolor attending the Dermatology and Venereology outpatient department of Dr B R Ambedkar Medical College and Hospital, Bangalore during a period of 6 months from January 2016 to June 2016. The diagnosis of Tinea versicolor was based on the clinical history and physical examination. This study was carried out in the Department of Microbiology, Dr B R Ambedkar Medical College and Hospital, Bangalore.

SAMPLE COLLECTION: The affected area was cleaned aseptically with 70% ethyl alcohol and allowed to dry. Skin sample was collected directly on glass slide using a pre flamed blunt scalpel from the edge of the lesion. [8]

MICROSCOPIC EXAMINATION: Preliminary direct microscopy was done by adding 2-3 drops of 10% KOH solution to the collected sample on the glass slide and a cover slip was placed. [8]

CULTURE STUDY: This was followed by culturing the skin sample on to antibiotic incorporated Sabouraud dextrose agar (SDA) media with chloramphenicol 50 mg/L and cycloheximide 500 mg/L with an overlay of sterile olive oil and incubated at 37°C for 4-5 days and the culture growth observed. The inoculated plates were observed for the growth of *Malassezia* for 4-8 weeks before negative results were reported. [1, 8] Since members of the genus *Malassezia* share similar morphology, CHROMagar*Malassezia* was used to further confirm isolates as *Malasseziafurfur*. [9]

BIO CHEMICAL TESTS: Further confirmation of *Malasseziafurfur* was done using the different biochemical tests including catalase test, bile esculin splitting test, urease test and sugar fermentation tests.

Catalase Test: A drop of 30% hydrogen peroxide was added to the colony isolate on a sterile glass slide with positive and negative controls. [8, 9, 10]

Bile Esculin Splitting Test: The β -glucosidase activity of *Malassezia* species was assessed by this test. Loop of fresh yeast was inoculated deeply in the esculin agar tube and incubated for 5 days at 30°C. The splitting of esculin was revealed by darkening of the medium. [8, 10]

Urease Test: Endogenous urease in the yeast cells is released into the test medium responsible for a positive reaction. The colonies were streaked on Christensen's Urease medium. [11]

Sugar Fermentation tests: Fermentation of sugars dextrose, xylose, lactose, maltose and mannitol were tested by inoculating the colonies into test tubes containing particular sugars with Andrade's indicator and Durham's tube and incubating the same at 37°C for 4-5 days. [12]

BIOFILM DETECTION: The detection of biofilm of the isolates were done by crystal violet (0.5%) staining of the smear followed by washing with deionized water. This was used to highlight amyloid that forms the infrastructure of biofilms. [1, 5]

RESULTS

KOH was positive for short hyphae and round yeast cells ("spaghetti and meatball" appearance) (Figure 1) in 72 cases (90%). Cultures were positive (Figure 2) for growth of *Malassezia* species in 78 of 80 samples (97.5%). Out of 78 positive cultures 24 isolates (30.76%) were confirmed to be *Malasseziafurfur* species.

The colony morphology described as buttery, cream or yellowish colonies (Figure 3). Gram stain of the culture growth was observed as gram positive oval, elongated cells, with unipolar budding, producing no pseudohyphae (Figure 4). LPCB (Figure 5) also showed budding yeast cells. CHROMagar*Malassezia* showed large pink and wrinkled colonies (Figure 6).

Biochemical reactions:

- Catalase Test: Positive test indicated by gas bubbles (Figure 7).
- Bile Esculin Splitting Test: No change in colour indicated as a negative test (Figure 8).
- Urease Test: Urease was hydrolysed (Figure 9).
- Sugar Fermentation tests: Dextrose and xylose was observed to be fermented without gas while lactose, maltose and mannitol were not fermented (Figure 10).

The majority of patients were of the age group 20-40 years constituting 65% of which males were 35% and females were 30%. Followed by the age group <20 years constituting 30% of patients and 5% of patients were of age group >40 years. On the whole, males (62.5%) were more affected than females (37.5%) by *Tinea versicolor* in this study (Table 1 and Graph 1).

90% of the samples were positive for KOH mount and culture. 7.5% of the samples were negative for KOH mount but positive for culture. 2.5% samples were negative for KOH mount and culture (Table 2).

Out of 80 clinical samples 24 isolates (30%) were identified as *Malassezia furfur* species. Biofilm (slime) was detected in all these isolates demonstrated by crystal violet staining showing aggregates of yeasts cells, the “dumps” and “streaming” both taking up the stain representing biofilm(Figure 11).

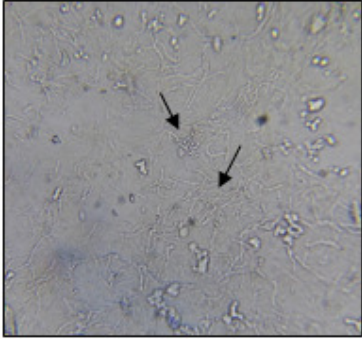


Figure 1: KOH mount
“Spaghetti and meatball”

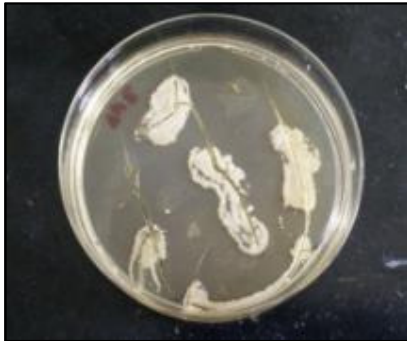


Figure 2: Culture of *Malassezia furfur* on
SDA plate with antibiotics and sterile
olive oil overlay



Figure 3: Creamy white buttery
colonies of *Malassezia furfur* growth
on SDA plate

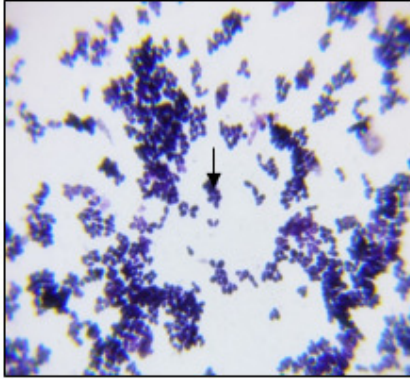


Figure 4: Gram Staining of *Malassezia furfur* showing oval unipolar budding yeast cells with no pseudohyphae

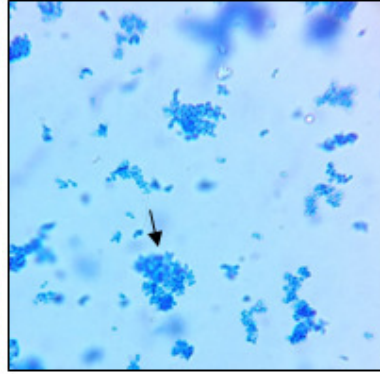


Figure 5: LPCB Mount of *Malassezia furfur* showing budding yeast cells

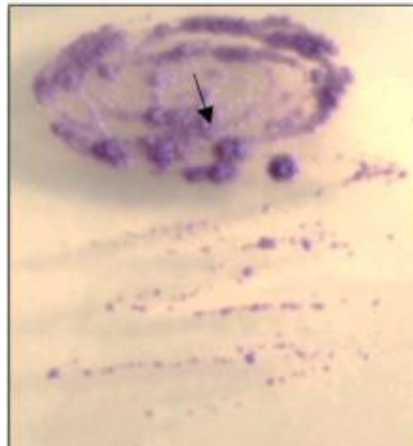


Figure 6: Colonies of *Malassezia furfur* on CHROMagar *Malassezia* showing large pink wrinkled colonies



Figure 7: Slide Catalase Test (C+: Positive control, T: Test, C-: Negative control)



Figure 8: Bile Esculin Splitting Test
(C+: Positive control, T: Test, C-:
Negative control)

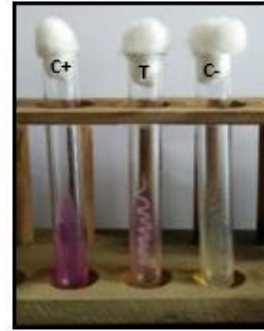


Figure 9: Urease Test (C+: Positive
control, T: Test, C-: Negative control)

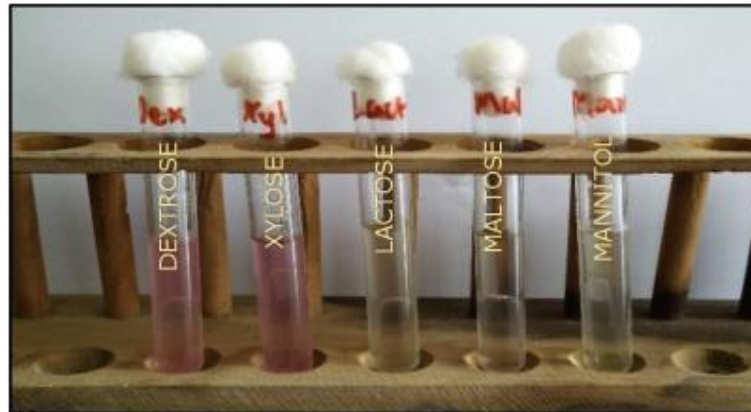


Figure 10: Fermentation of Sugars [(Dextrose, Xylose)- Fermented with no gas,
(Lactose, Maltose, Mannitol)- Not fermented]

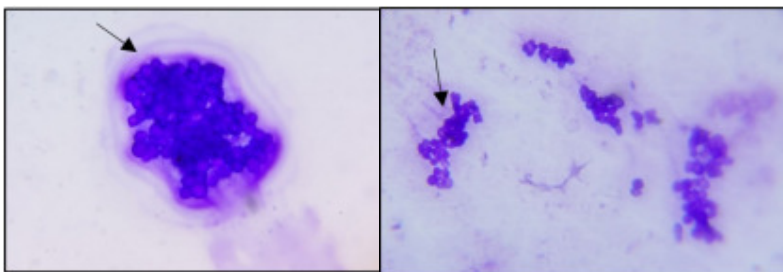


Figure 11: Biofilm formation demonstrated in *Malassezia furfur* species by crystal violet staining

Table.1 Age and Gender wise Distribution of Clinical cases of Tinea versicolor

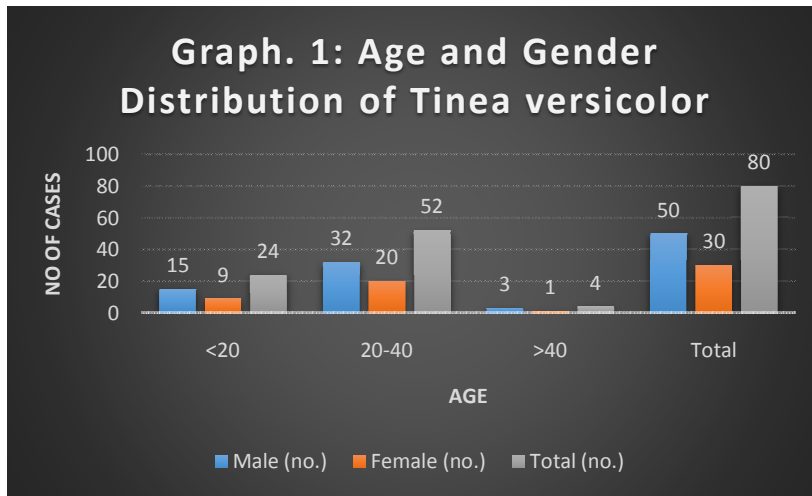


Table.2 Comparison of KOH positivity by slide and culture

KOH wet mount	Culture	Total (no.) %
Positive	Positive	72 (90%)
Negative	Positive	06 (7.5%)
Negative	Negative	02 (2.5%)
		80 (100%)

DISCUSSION

In our present study with 80 clinical samples, 72 cases (90%) were positive for fungal elements by KOH mount and culture, 6 cases (7.5%) of the samples were negative for fungal elements by KOH mount but positive for culture and 02(2.5%) of samples were negative for fungal elements by KOH mount as well as culture. Also in our present study we observe that out of 80 clinical samples 24 isolates were detected to be *Malassezia furfur* and all those isolates showed positive staining with crystal violet demonstrating biofilm in them.

Similarly, in a study conducted by Allen HB et al [1] showed that out of 24 samples processed 20 samples showed growth of *Malassezia furfur* and all were positive for biofilm formation demonstrated by crystal violet staining. Cannizzo et al. [13] and Figueredo et al. [5] independently found that the lipid dependent *Malassezia pachydermatis* derived from normal and infected skin samples had the ability to create biofilms in vitro. A study done by Thayikkannu AB et al. [14] demonstrated biofilm formation in all 10 strains of *Malassezia furfur* isolated from clinical samples.

Despite the fact that the majority of our patients were male, Tinea versicolor has not been shown to have a predilection for either sex overall [15]. By demonstrating the presence of amyloid protein (easily visible) in the *Malassezia* slime as evidenced by positive staining with crystal violet in all positive cultures, we have shown that *Malassezia* can produce biofilm. We hypothesize that biofilm formation contributes to the pathogenicity of *Malassezia* species in Tinea versicolor.

The standard treatment for Tinea versicolor consists of generally topical and sometimes oral antifungals in chronic cases [2]. Topical anti-fungal drugs such as azoles are the first-line agents used for treatment of *Malassezia* due to its predilection for the stratum corneum. Oral agents may also be implemented in severe cases. Azoles act as a fungistatic,

whereas adding selenium sulfide or zinc pyrithione, acts a biofilm-disperser and helps to decrease numerous *Tinea versicolor* relapses after treatment. [1]

There is a need to address the persistence and difficulty in dismantling biofilms. It is therefore necessary to utilize biofilm dispersing agents periodically in treating *Tinea versicolor* if we have to successfully prevent chronic disease. There are a wide range of agents that target different stages of the biofilm maturation process, particularly adhesion and dispersion [1]. Although yeast biofilms are similar to bacterial biofilms, their extracellular matrix is not similar and therefore may not be susceptible to the same biofilm-dispersal agents of bacterial biofilms [15]. Less bacteria-specific biofilm dispersal treatments such as photodynamic therapy are being designed for the elimination of fungal biofilms[1]. These may have applications in eradicating *Tinea versicolor* in the near future.

CONCLUSION

The present study helps to conclude that *Malassezia furfur* isolated from clinically diagnosed cases of *Tinea versicolor* demonstrate biofilm formation. Thus it concludes that presence of biofilm can be demonstrated by simple tests like crystal violet staining in addition to preliminary KOH mount and a culture for confirmation especially in chronic cases or in cases with treatment failure. It can be considered that biofilms have contributed to the pathogenesis of *Tinea versicolor* the yeast being a normal flora of skin. Biofilms have also been regarded as a cause for chronic *Tinea versicolor* in spite of repeated treatment with antifungal agents necessitating the use of biofilm dispersing agents in treatment regimen for complete cure.

Acknowledgements:

It is with great privilege and respect that I express my most cordial and humble thanks to my respected teacher and guide, **Dr. (Mrs). Saroj Golia** MD, Professor and HOD Department of Microbiology, Dr.B.R.Ambedkar Medical College Bangalore. I owe my indebtedness for her unparalleled and excellent guidance, continuous encouragement, in working on this study. I feel fortunate to work under her.

I express sincere appreciation to my senior colleagues **Dr. Jyoti and Dr. Suhani S Manasa** for their contribution in the study and their support and co-operation.

I am extremely grateful to all the technical staff, **Mrs. Annamma A M, Mr. Govinda M, and Mrs. Sushila S**. I am also thankful to the attenders **Mr. Doddabeeraiah and Mr. Thiruneshan**.

I express deep sense of gratitude to all the patients who co-operated for the study.

I thank my mother and uncle for their moral support and encouragement.

I, finally thank Almighty **God** for granting me blessings, good health, wisdom and strength to take up this study and bring it to its logical conclusion.

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