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Biofilm Formation by *Malassezia Furfur/Ovale* as a Possible Mechanism of Pathogenesis in Tinea Versicolor

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Abstract

Malassezia species exist as normal flora on human skin but can convert to a pathogenic state in response to a number of host and environmental factors ultimately resulting in tinea versicolor (TV). Biofilm formation represents one of the main mechanisms by which microorganisms maintain viability in hostile environments. We have shown that *Malassezia furfur/ovale* cultured from patients with active TV can produce biofilms in vitro and in vivo. Exposure of *Malassezia* to sweat in vivo is the likely trigger for biofilm formation (as it is in atopic dermatitis); we believe this biofilm formation is potentially responsible for both the pathogenesis and the chronicity of this infection. This gives rationale to biofilm-dispersing agents, such as selenium sulfide, as important components in the standard TV treatment regimen. Periodic use of such agents would conceivably prevent reinfection.

Keywords: Tinea versicolor; Biofilm; Crystal violet

Introduction

The most common cutaneous yeast/fungal infection in the world, tinea versicolor (TV), occurs when *Malassezia furfur* and *ovale* (lipophilic yeast forms now considered to be the same organism), which are part of the normal skin flora, assume a pathogenic state within the epidermis. When this occurs, patients will present with hypopigmented and/or hyperpigmented scaling lesions on the upper trunk even in patients with normal immunity. Infection may become chronic despite repeated treatment [1]. *Malassezia* species have also been implicated in the pathogenesis of seborrheic dermatitis and *Malassezia* folliculitis [2].

Biofilm formation serves as a protective mechanism for many microorganisms as it allows for evasion of immune surveillance, provides a safe haven for proliferation, and acts as a barrier against antimicrobial agents [3,4]. Although traditionally associated with bacteria, many fungi and yeasts like Trichophyton, Candida, and *Malassezia* can form biofilms [5]. Interestingly, the interaction between fungi and bacteria appears to be one of the strongest environmental factors influencing biofilm formation due to quorum-sensing molecules and increased co-aggregation after initial colonization by one species [6].

Biofilms are generally composed of a matrix of extracellular polysaccharides, amyloid, DNA, and adhesive fibers that permits the permanent adhesion of the colony to biologic and non-biologic surfaces including healthy skin [7,8]. Fungal biofilms also contain yeast and hyphal forms which are thought to contribute to adherence as well as chitins and β-1,3 glucan carbohydrates [5]. The matrix 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 [4,7]. It has been suggested that biofilms may be the source of relapse of so-called "noninfectious" diseases such as acne vulgaris, atopic dermatitis, miliaria, and onychomycosis after the termination of antimicrobial treatment [8]. Nondividing persister cells residing within the biofilms may be responsible for reseeding biofilms post-treatment as they are immune to most antimicrobial agents and can deactivate their apoptosis pathway [7,9].

There have been very few studies examining the ability of *Malassezia* yeasts to form biofilms in vitro and in vivo although this has been demonstrated in some yeasts, particularly Candida [8]. Cannizzo et al. and Figueredo et al. independently found that the lipid-independent dermatophyte *Malassezia pachydermatis* derived from normal and infected skin had the ability to create biofilms in vitro [2,3,10]. Herein, we hypothesize that biofilm formation by *Malassezia* species is potential mechanism behind its transformation from normal skin flora to pathogen and subsequent expression as TV.

## Materials and Methods

Ethics Approval

Approval for this study was granted by the Drexel University College of Medicine (DUCOM) Institutional Review Board.

Sample Collection and Processing

The diagnosis of TV was based on the clinical history and physical examination. (Figure 2) Skin scrapings were obtained from 24 patients with active TV presenting to the Drexel University College of Medicine Dermatology Clinic. Samples were taken for potassium hydroxide (KOH) preparations and for Crystal violet staining. They were also directly inoculated on antibiotic-containing agar culture medium. Of the 24 patients, 19 were from males and 5 from females ranging in age from 16 to 48-years old. Patient demographics can be found in Table 1. Samples were taken from various anatomic sites including chest, back and neck. 20 control samples were also acquired from the chest and back of the same patients.

Age range (years)	Sex	M:F ratio	Co-morbidities
16-48	Male	3.8:1	Morbid obesity
	Female		*Acanthosis nigricans*
			Diabetes mellitus
			Hypertension

Table 1: Demographics of 24 patients with suspected tinea versicolor

Figure 1: Proposed mechanism by which tinea versicolor becomes chronic.
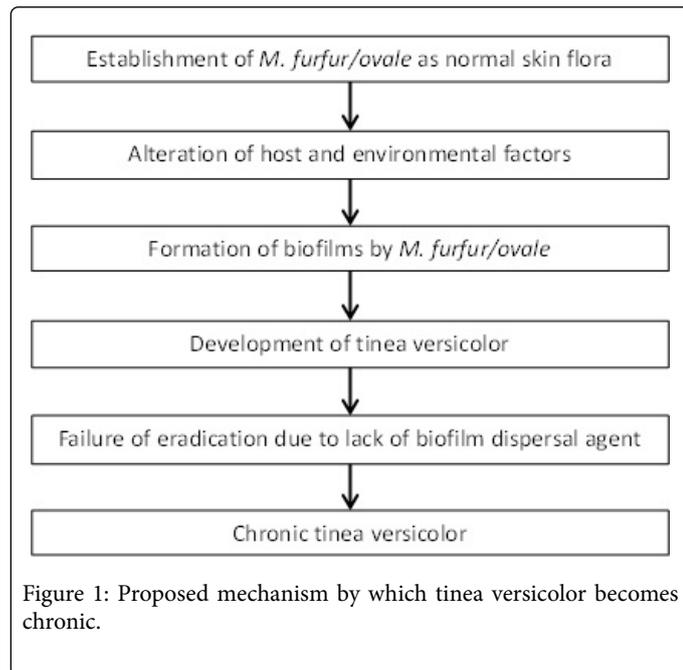
Sterile olive oil was added to each culture to enhance growth. These culture plates were incubated for 8-12 weeks at room temperature. Colonies were then stained with crystal violet. This was used to highlight amyloid that forms the infrastructure of biofilms. The samples were then preserved for future genetic analysis.

Figure 2: Clinical presentations of hypo and hyperpigmented TV.
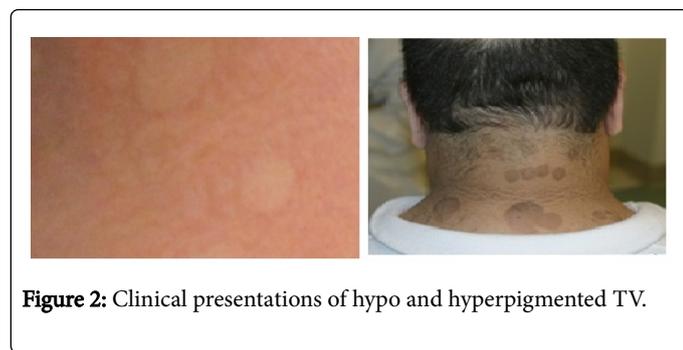
Results

KOH was positive for short hyphae and spores ("spaghetti and meatballs") in all cases (Figure 3) and in none of the controls. Crystal violet staining was positive showing aggregates of organisms in all (Figure 4). Cultures were positive for growth in 20 of 24 samples. Biofilm (slime) was present in those positive cultures; this is demonstrated in Figure 5 Crystal violet staining was present in all positive cultures (Figure 6).
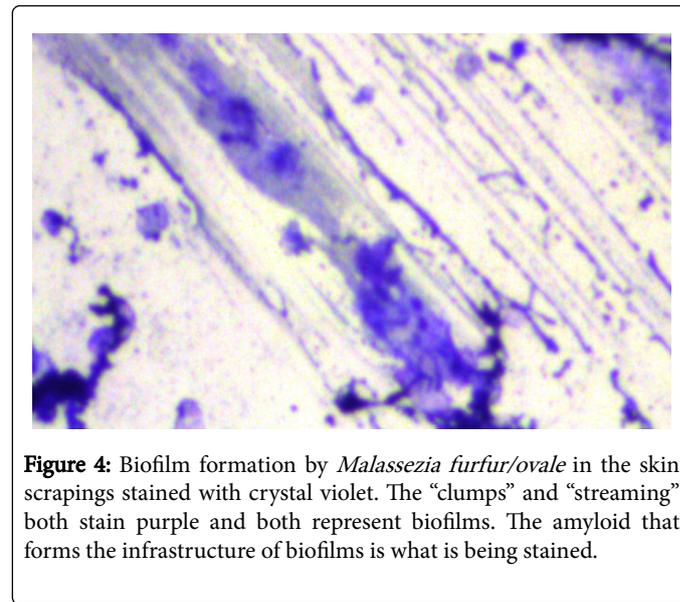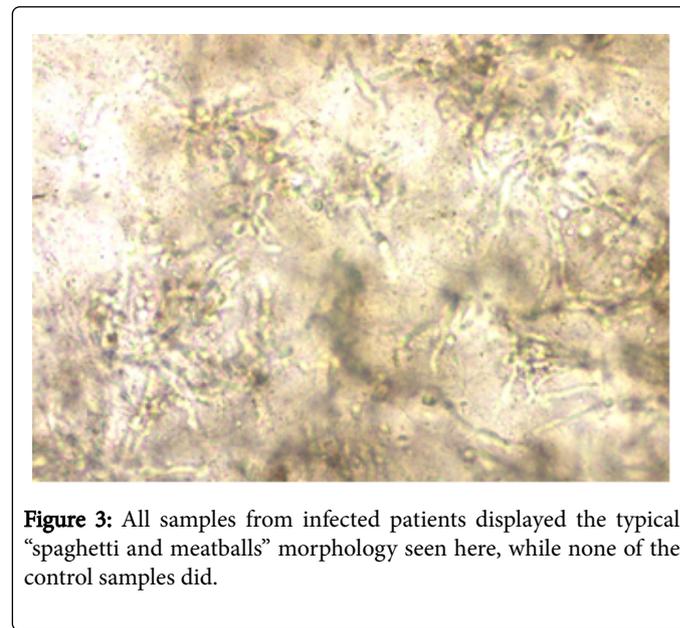

Figure 3: All samples from infected patients displayed the typical "spaghetti and meatballs" morphology seen here, while none of the control samples did.

Figure 4: Biofilm formation by *Malassezia furfur/ovale* in the skin scrapings stained with crystal violet. The "clumps" and "streaming" both stain purple and both represent biofilms. The amyloid that forms the infrastructure of biofilms is what is being stained.

Figure 5: Culture of TV revealing biofilm (slime)

Both of these conditions are known to contribute to immunosuppression which may explain their prevalence in our TV patients [13,14]. Despite the fact that the majority of our patients were male, TV has not been shown to have a propensity for either sex overall [15]. Exogenous factors such as sunlight, corticosteroids, and oil-based products encourage the growth of *Malassezia* yeast forms [12].

In response to the aforementioned factors, *Malassezia* enters a pathogenic state and clinically manifests as TV. Hypopigmentation results from the secondary effect of dopa-tyrosinase inhibition by azelaic acid that is produced by lipases present in *Malassezia* [12]. However, the main action of these phospholipases is to permit adhesion to the skin, the first step in infection. The timing and 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. The quantity of biofilm generated by *Malassezia* pachydermatis is directly proportional to phospholipase levels.
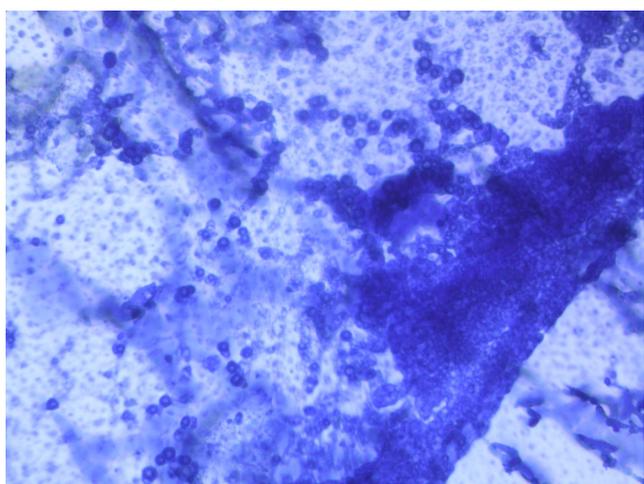

Figure 6: All positive cultures demonstrated staining for crystal violet indicating the presence of amyloid in the clumps of biofilm

Discussion

Given the presence of amyloid protein in the (easily visible) *Malassezia* slime as evidenced by positive staining with crystal violet in all positive cultures, we have shown that *Malassezia furfur/ovale* can produce biofilms because amyloid forms the infrastructure of biofilms. We hypothesize that biofilm formation contributes to the pathogenicity of *Malassezia* spp. in TV. We previously found that biofilm-producing staphylococci comprise the environmental component of the double-hit hypothesis in atopic dermatitis (AD). The development of "subclinical miliaria" in AD is due to the obstruction of eccrine sweat ducts by biofilms produced by staphylococcus species. Mutations in filaggrin (or other) genes represents the first hit that initially predisposes patients to AD [11]. TV may follow a comparable double-hit hypothesis. Similar to filaggrin mutations in AD, patients with a propensity for developing TV may have a genetic component that predisposes them to disease, whether it is immunosuppression or hyperhidrosis. The second hit is environmental – in this case, the presence of *Malassezia* spp. in anatomically vulnerable areas of the skin. Other exogenous factors may then trigger the pathogenic pathway and result in clinical disease in those who are susceptible to TV. Figure 1 describes a possible pathway through which *Malassezia* biofilm formation may cause chronic TV.

Malassezia infection is first established when a subject comes into contact with an infected individual or object with localization of the fungi to the stratum corneum [1,12] There are a number of host and environmental factors that promote the development of TV. Humidity is an important element in disease pathogenesis as evidenced by an increased rate on infection in tropical climates compared to temperate areas.

Immunosuppression, hyperhidrosis, and hereditary predisposition on the host side increase the risk of developing clinical disease [1]. Sweat has been shown to be an important instigator of biofilm production in diseases like AD due to both the water and salt components [11]. In our population, 40% of the patients were morbidly obese and 25% had diabetes mellitus

This suggests that the two mechanisms may act in concert to afford microorganism's enhanced protection from the host immune response and lead to clinical worsening of disease [3]. Phospholipases also induce an inflammatory response and can harm the cell membrane [3]. For most dermatophytes, for example, a Th1-predominant immune response is evoked but this can be downregulated by metabolites produced by *Malassezia*. The immune response is further buffered by localization of the organisms to the stratum corneum where immune detection can be bypassed [12]. This leads to the main difference in the biofilms produced in TV versus AD: they, apparently, do not trigger the innate immune system and thus do not initiate any pathological inflammation. This would lead to the lack of pruritus present in most patients.

The standard treatment regimen for TV consists of topical and oral antifungals [1]. Topical medications such as azoles, selenium sulfide, and zinc pyrithione constitute first-line agents given *Malassezia*'s predilection for the stratum corneum [16]. Oral agents may also be implemented [17]. Azoles act as a fungistatic, whereas selenium, not only is a biofilm-disperser (see below), but it also helps to increase numerous TV relapses after treatment. This is likely due to the failure of our current regimen to address the persistence of and difficulty in dismantling biofilms. It is therefore necessary to utilize biofilm-dispersing agents periodically in treating TV if we are to successfully prevent chronic disease. The extent of the disease has been shown by in vivo Gram staining (crystal violet) to be very dramatic [18].

There are a broad range of agents that target different stages of the biofilm maturation process, particularly adhesion and dispersion. Silver has long been known to act as a bactericidal through its interference in replication and cellular respiration. Recent studies on silver nanoparticles have proven their utility in preventing biofilm formation by Staphylococcus epidermidis and Pseudomonas aeruginosa [5]. This lends credence to the use of selenium or heavy metal-containing medications in TV for their ability to disperse biofilms [19,20,21]. Fluoroquinolones are known to be effective against P. aeruginosa biofilm formation through their ability to induce apoptosis in normally apoptotic-resistant persister cells [9,22]. Newer molecules such as dispersin B, alginate lysate, sodium nitroprusside, and diguanylate cyclase enzymes have been found to be effective bacterial biofilm dispersal agents in vitro. The translation of these agents to the in vivo setting has not yet been investigated and so their clinical utility remains uncertain [4]. Although yeast biofilms form similarly to bacterial biofilms, their extracellular matrix is dissimilar and therefore may not be susceptible to the same biofilm-dispersal agents being studied in the context of bacterial biofilms [5]. Less bacteria-specific treatments such as photodynamic therapy are being designed for the elimination of biofilms, particularly in acne vulgaris [8] These may have applications in eradicating TV in the future.

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