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# Clinico-Mycological Study of Fungal Biofilms in Recalcitrant Onychomycosis

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## Abstract

**Objective:** Studying the incidence of different predisposing factors of onychomycosis and the ability of fungal isolates to form biofilms.

**Background:** Onychomycosis chronicity is referred to difficulties in eliminating the causative pathogens. Biofilms have become focus of era and considered to be one of the globally important causes of chronic recalcitrant infections. Fungi implicated in onychomycosis have the ability to form biofilms "surface-attached multi-cellular communities". Biofilm associated fungi acquire higher adaptive ability to overcome stressful conditions, higher antifungal resistance and evasion of host defensive systems. Moreover, they express community-based differential genes and more virulence activities.

**Methods:** Fifty-four military male patients with onychomycosis were enrolled in this study from March, 2015 to October, 2015. All patients participating signed an informed detailed consent, full history taking, general and local examination and clinical photography. Nail specimen were collected to be examined by using direct light microscopy of 20%KOH nail mounts, cultured on different fungal agars media and to evaluate the ability of isolates to form biofilms as well as quantity by using (RPMI)-1640 buffered with (MOPS) (HiMedia, India) and (XTT) solution (Sigma-aldrich, USA), respectively.

**Results:** While fungal elements were observed only in 51% of 20%KOH nail mounts by direct light microscopy, all specimen gave positive culture results (26 *C. albicans*, 26 *T. rubrum* and 2 *M. canis*). Twenty-eight isolates (19 *C. albicans* and 9 *T. rubrum*) were able to form biofilms *in vitro*. Biofilm forming ability was significantly related to positive history of nail exposure to high humidity micro environments (p=0.05), repeated or rough nail trauma (p=0.006), diabetes mellitus (p=0.003) and past history of receiving antifungal agents before the present study (≥ 5 months according to the exclusion criteria) (p=0.006).

**Conclusions:** We found significant relations between the ability of fungi to form biofilm and factors that paly role in recalcitrant onychomycosis infections such as repeated minor nail trauma, high micro environmental humidity, complications of diabetes mellitus and antifungal misuse (type, dose and/or duration).

**Keywords** Military boots; Dermatology; Biofilm; Antifungal; Dermatophyte fungi; Extracellular matrix; Nail; Onychomycosis

## Introduction

Onychomycosis is a fungal infection of nails [1]. It accounts for half of all nail disorders and its prevalence has been increasing. Almost, one third of patients with cutaneous fungal infections were found to have concomitant onychomycosis. Toenails are more likely to be infected than fingernails in male patients and *vice versa* [2].

It may cause nail disfigurement, produce physical and occupational limitations especially in patients who are providing food services or drinks to the public and may lead to psychosocial and emotional imbalance with a significant negative impacts on quality of life and self-image [3-5].

In onychomycosis, several factors including firm adherence of fungi to the nail plate, presence of dormant fungal elements, wearing high sealed boots or repeated hands water immersion and difficulty of fungal eradication despite long courses of systemic antifungal agents suggest that biofilms may be implicated in recalcitrant onychomycosis [6,7].

The latter has directed researchers towards the evaluation of the fungicidal activity of both light and laser treatment in recalcitrant onychomycosis of human nail fragments that have been used successfully *in vitro* as a substrate for fungi to form biofilms [7].

Fungi are traditionally understood as existing in the environment as planktonic organisms; however, recent advancements in microbiology suggest that fungi form biofilms; a special environment surrounded by a matrix of extracellular polymers.

Fungal biofilms show higher antifungal resistance and evasion of host defensive systems. Moreover, they express community-based differential genes and more virulence activities [1,8].

## Patients and Methods

### Patients

This study included 54 military male patients presented with clinical nail signs of onychomycosis to Dermatology outpatient clinic of Kobry El-Kobba Armed Forces Medical Complex from March, 2015 to October, 2015. All patients participating signed an informed detailed consent, full history taking, general and local examination and clinical photography. Patients who received topical anti-fungal therapy in the preceding one week or systemic anti-fungal therapy in the preceding <5 months were excluded. The study was approved by the ethical committee, Ain Shams University.

## Methods

### Nail specimen collection

After disinfecting the targeted nail with 70% alcohol, nail samples were collected by subungual and superficial nail scraping then nail clipping according to Elewski [9].

## Direct light microscopic examination

According to Shenoy [10], light microscopic examination of prepared 20% KOH nail mounts at different lens powers (X5, X10 and X40) were considered positive when microscopic morphological fungal features were seen (Figure 1).

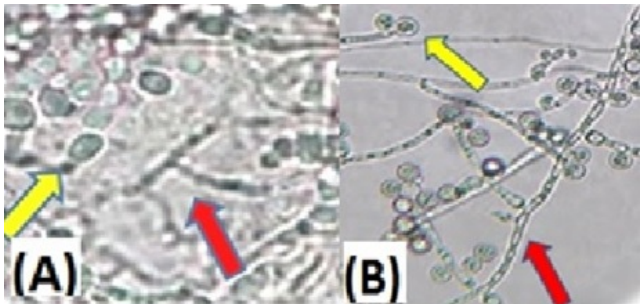


**Figure 1:** (A) Direct KOH examination under light microscopy of nail specimen showing fungal elements of dermatophytes (pseudohyphae "red arrow" and conidia "yellow arrow") (X10), (B) Direct KOH examination under light microscopy of nail specimen showing fungal elements of candida (pseudohyphae "red arrow" and Chlamydoconidia "yellow arrow") (X10).

## Fungal culture and identification

Specimens were inoculated in four to five sites in 3 different culture plates containing sabouraud's dextrose agar medium+chloramphenicol for isolation of non dermatophytic moulds and yeasts, sabouraud's dextrose agar medium+chloramphenicol+cyclohexamide for isolation of dermatophytes and yeasts and chromogenic candida agar medium for isolation of candida species. The first two cultures were incubated at 25˚C under aerobic conditions and observed for growth in each day of the next 2-4 weeks [11]. The chromogenic candida agar medium was incubated at 37˚C under aerobic conditions and observed for growth in each day of the next 48-72 hours [12]. The colonies were identified by their rate of growth and macro-morphology (Figure 2) [9].

**Figure 2:** *C. albicans* in green colonies on chromogenic candida agar.

## Biofilm formation

Isolates were enhanced by using Roswell Park Memorial Institute (RPMI)-1640 buffered with 3-(N-morpholino) propanesulfonic acid (MOPS) (HiMedia, India) [13]. RPMI-MOPS cell suspension was inoculated in 96-well polystyrene plates with diluted antifungal agents (Figure 3) [14].

**Figure 3:** *T. rubrum* colony on SAD+C+C.

## Biofilm quantification

The inoculated RPMI-MOPS cell suspension was removed and the adherent cells were washed three times with PBS. These were air-dried and 100 µl of sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) - 5 - [(phenylamino) -carbonyl]-2H-tetrazolium inner salt (XTT) solution (Sigma-aldrich, USA) was added to each well and to appropriate control wells to measure background XTT reduction levels. The colorimetric change in the XTT reduction assay directly correlates

with the metabolic activity of the biofilm. The plates were incubated in the dark for 3 h at 37˚C and the colour change "optical densities" (OD) of stained adherent fungal films were read after 30 minutes using a 490 nm filter in a micro plate reader (FLUOStar Optima plate reader) (BMG Labtech, UK). Negative values were presented as zero, while positive values were presented as weak biofilm producer (+1), moderate biofilm producer (+2) and strong biofilm producer (+3) (Figures 4 and 5) [15].

**Figure 4:** *M. canis* colony (fluffy white colony) on SAD+C+C.

**Figure 5:** *M. canis* colony (reverse deep yellow) on SAD+C+C.

## Data management and analysis

The collected data was revised, coded, tabulated and introduced to a PC using Statistical Package for Social Science software (IBM Corp. Released 2014. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: USA. IBM Corp.). Data was presented and analyzed according to the type of data obtained for each parameter while p-value represents the significance level.

## Results

Among nail specimens, 28/54 prepared KOH nail mounts (51.8%) were positive by light direct microscopy while all cultured specimens were positive as presented in Table 1. Age distribution and duration of onychomycosis are presented in Table 2. As regards positive biofilm forming ability, 28 out of 54 culture samples were positive with variable strength (Table 3). In the present study, 92.8% of positive biofilm producing organisms were isolated from cases with positive history of exposure to humid environments. In addition, 53.5% of positive biofilm producing organisms were isolated from patients with D.M. The presence of D.M showed statistically highly significant differences as regards biofilm producing ability and quantification while exposure to increased humidity environments showed significant difference as regards the biofilm ability but not quantification of biofilms formed as presented in Table 4. Moreover, 78.5% of positive biofilm producing organisms are isolated from patients who used oral antifungals ≥ 5 months prior to the study and failed to treat onychomycosis. This result showed statistically highly significant difference as regards biofilm producing ability of isolated organisms and biofilm quantity as presented in Table 5. The presence of nail trauma, diabetes mellitus and previously received antifungal agents showed statistically highly significant differences as an independent predisposing factors affecting biofilm formation (Table 6).

| **Isolates genera** | | **Number** |
|---|---|---|
| *Candida* species (n=26) | *C. albicans* | 26 |
| Dermatophytes species (n=28) | *T. rubrum* | 26 |
| | *M. canis* | 2 |

**Table 1:** Identified isolates genera

| | **Minimum** | **Maximum** | **Mean** | **± SD** |
|---|---|---|---|---|
| Age (Years) | 20 | 79 | 56 | 17.66 |
| (Years) Disease duration | 6 months | 10 years | 28 months | 20 months |

**Table 2:** Age distribution and duration of onychomycosis

| | | *C. albicans* | *T. rubrum* | **P-value Significance** |
|---|---|---|---|---|
| Positive Biofilm Producing Ability | Number (n=28) | 19 | 9 | 0.007 Highly Significant |

| | | | | |
|---|---|---|---|---|
| Biofilm quantification | percentage | 68% | 32% | 0.005 Highly Significant |
| | Weak (+1) | 5 | 8 | |
| | Moderate (+2) | 13 | 1 | |
| | Strong (+3) | 1 | 0 | |

**Table 3:** Isolated organisms and biofilm producing ability and quantity.

| | | High humidity | P-value Significance | Diabetes Mellitus | P-value Significance | Repeated or rough nail trauma | P-value Significance |
|---|---|---|---|---|---|---|---|
| **Positive Biofilm Producing Ability** | Number (n=28) | 26 | 0.05 Significant | 15 | 0.003 Highly Significant | 18 | 0.006 Highly Significant |
| | percentage | 92.80% | | 53.50% | | 64.2 | |
| **Biofilm quantification** | Weak (+1) | 12 | 0.28 Non-Significant | 5 | 0.009 Highly Significant | 7 | 0.03 Significant |
| | Moderate (+2) | 13 | | 9 | | 10 | |
| | Strong (+3) | 1 | | 1 | | 1 | |

**Table 4:** Predisposing factors and biofilm producing ability and quantity

| | | Previous antifungal treatment | P-value Significance |
|---|---|---|---|
| Positive Biofilm Producing Ability | Number (n=28) | 22 | 0.006 Highly Significant |
| | percentage | 78.50% | |
| Biofilm quantification | Weak (+1) | 5 | 0.016 Highly Significant |
| | Moderate (+2) | 17 | |
| | Strong (+3) | 0 | |

**Table 5:** Treatment received prior to study and biofilm producing ability and quantity

| | 95% C.I for odds ratio | | P-value | Significance |
|---|---|---|---|---|
| | Lower | Upper | | |
| High Humidity | 0.8934 | 25.6763 | 0.0675 | Insignificant |
| Nail Trauma | 1.5296 | 15.6051 | 0.0074 | Highly Significant |
| Diabetes Mellitus | 1.7318 | 23.2558 | 0.0053 | Highly Significant |
| Previous Treatment | 1.519 | 16.464 | 0.008 | Highly Significant |

**Table 6:** Independent predisposing factors affecting biofilm producing ability.

## Discussion

It is commonly reported that onychomycosis prevalence increases in elderly patients and about 20% of the populations aged over 60 years are reported to have onychomycosis that may persist for months or years [5,16]. Elderly patients are more liable to have diseases that predispose to onycho-mycosis such as: D.M and vascular circulatory disorders. In addition, the net result of frequent repeated nail traumas throughout life may also play a rule, as trauma causes distortion of the three nail layers making nails liable to be colonized by fungal organisms [17].

Moreover, increased onychomycosis incidence in elderly may be attributed to the slower growth rate of nails with aging and so, organisms are not cleared away in a regular manner and nail changes remain for a longer time.

While potassium hydroxide mounts is not highly specific and may fail to reveal fungal elements in some cases, they are still considered a

rational investigation in cases with onychomycosis as it is an easy and inexpensive useful method in identification of fungal elements [18]. Shenoy et al. and Raghavendra et al. reported the presence of fungal elements among direct microscopic examination of nail clippings mounted with 40% KOH in 53% and 55.33% of cases, respectively. On contrary, false-negative results were relatively high in other studies [19]. This may be referred to a defect in the preparatory technique or undetectable fungal elements surrounded by undissolved matrix of extracellular polymers which consist the cornerstone of biofilm. However, nail specimen cultures in the present study showed highly positive results for the presences of fungal organisms which are in accordance with Raghavendra et al. The isolated fungal results in our study are compatible with many other studies reported that dermatophytes, mainly *Trichophyton rubrum* "*T. rubrum*", as the most common fungal organisms involved in onychomycosis followed by yeasts, mainly *candida albicans* "*C. albicans*", in many countries as Great Britain, Egypt and India [5,13,20-24]. Biofilm has become the focus of the era and is considered to be one of the important causes of chronic recalcitrant infections globally. It is important to note that many factors, such as the nature of the host surfaces, host immunity interactions and quorum sensing, can affect biofilm production *in vivo*. Thus, intensity (quantity) of biofilm produced *in vitro* could be quite less than that expected *in vivo*.

The isolated organisms with biofilm producing ability in our study accounted 52% of isolates. Producing organisms, *C. albicans* and *T. rubrum*, have shown highly significant differences in regards to organism species with biofilm producing ability and quantity (p=0.007 and 0.005, respectively). Thus, biofilms formation and quantities are significantly more common in *C. albicans* than *T. rubrum* (Table 3).

According to our study, 92.8% of organisms with positive biofilm producing ability are isolated from patients with nails being exposed to high humid environments. Results showed a significant difference (p=0.05). This is in accordance with studies reported that temperature and humidity conditions stimulate fungal genes of biofilm formation 25. This may be explained by the effect of prolonged wearing of high sealed military boots in most of our patients. However, results showed no significant difference (p=0.280) as regards biofilms quantities *in vitro*.

Exposure to minor nail trauma in our study accounted 72% of cases with biofilm producing fungal organisms and showed a high significant difference for both biofilm formation (p=0.006) and biofilm quantity (p=0.03). In addition, repeated minor nail trauma as an independent predisposing factor for biofilm producing ability was highly significant; odd ratio (p=0.0074).

Repeated minor nail trauma results in localized loss of nail cuticle and tissue disintegration that may provide ideal sites for biofilm formation and induce micro-RNA expression in host tissues to silence tight junction protein functions. Tight junction proteins are critical for the proper maintenance of skin barriers, and their malfunction may further promote biofilm formation [25-29].

In our study, 53.5% of organisms with positive biofilm producing ability were isolated from patients presented with D.M and showed a high significant difference with the ability of fungi to produce biofilms (p=0.003), biofilm quantity (p=0.01) as well as a highly significant difference as an independent predisposing factor; odd ratio (p=0.0053). Diabetes may enhance the ability of biofilm formation indirectly as it causes both vascular insufficiencies and weak immune response. In addition, D.M. related complications, such as peripheral neuropathy, cataract, retinopathy and/or obesity, may lead to poor maintenance of foot hygiene (i.e. less physical removal of biofilms and infected tissue), and dystrophic nails may lead to minor skin trauma followed by cracks [28-30].

Antifungal agents may act as signaling molecules that regulate biofilm formation upon exposure of an organism to a sub-minimum inhibitory concentration. Therefore, drug susceptibility should be tested on organism in its biofilm mode and not planktonic mode to avoid treatment failure by increased antifungal tolerance and to prevent signal triggering of biofilm formation [31,32]. There was a highly significant correlation between the previous use of antifugal agents and the ability of fungi to produce biofilms (p=0.006) as well as biofilms quantities (p=0.016). In addition, previous antifungal use as an independent predisposing factor for biofilm producing ability was highly significant; odd ratio (p=0.008). About 78.5% of positive biofilm producing organisms were isolated from patients who had used antifungals ≥ 5 months prior to the present study. However, antifungal agents had failed to treat their onychomycosis.

## Conclusion

We found significant relations between the ability of fungi to form biofilm and factors that paly role in recalcitrant onychomycosis infections such as repeated minor nail trauma, high micro environmental humidity, complications of diabetes mellitus and antifungal misuse (type, dose and/or duration). However, further prospective study on larger population size is recommended in order to establish a definite correlation between biofilm forming ability and clinical predisposing factors of onychomycosis.

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