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In vitro biofilms and antifungal susceptibility of dermatophyte and non-dermatophyte molds involved in foot mycosis

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Summary

Background: Tinea pedis and onychomycosis are among the commonest fungal diseases in the world. Dermatophytes and, less frequently, non-dermatophyte molds are etiological agents of foot mycosis and could be able to form biofilms. Fungal biofilm has demonstrated increasing drug resistance **Objectives:** This work aims to evaluate *in vitro* the ability to form biofilm and the susceptibility to antifungal drugs of sessile dermatophytes and non-dermatophyte molds involved in foot mycosis. **Methods:** Thirty-six dermatophytes and non-dermatophyte molds isolated from Tunisian patients with foot mycoses, and identified with MALDI-TOF have been tested. MICs of fluconazole, econazole, itraconazole, terbinafine and griseofulvin were carried out using CLSI broth microdilution method. The ability to form biofilm and antifungal activities of drugs against fungal biofilm formation has been quantified by Crystal Violet and Safranin Red staining. **Results:** Biomass quantification revealed that all species studied were able to form biofilms *in vitro* after 72h. Fluconazole, econazole, itraconazole and terbinafine inhibited fungal growth with MIC values ranging from 0.031 to >64 µg ml^{-1}. The best antifungal activity has been obtained with terbinafine against *Fusarium solani.* Econazole showed the highest activity against fungal biofilm formation. **Conclusion:** These findings can help clinicians to develop the appropriate therapy of foot mycosis.

Introduction

Tinea pedis and onychomycosis are among the commonest fungal diseases in the world; these affect the elderly, children and adults [1-3]. The most frequently identified fungi are dermatophytes. *Trichophyton rubrum* is the most common specie but various non-dermatophyte molds (NDMs) are also isolated from diseased nails such as *Scopulariopsis brevicaulis, Fusarium* spp., *Aspergillus* spp. [4-6]. Among the known *Aspergillus* spp., *A. versicolor* and *A. sydowii* are the species most commonly associated to foot infections, including onychomycosis, other species like *A. candidus, A. fumigatus, A. niger, A. flavus, A. terreus, A. ochraceus and A. sclerotiorum*

are also incriminated [7,8]. *Fusarium solani* and *Fusarium oxysporum* are etiological agents of onychomycosis caused by *Fusarium* species [9].

These infections are considered as an important public health problem, constituting a large bulk of cases attending the dermatology departments and this can be due to the high prevalence, long-term therapy and difficult eradication of recurrent chronic nails infection[10-12].

The treatment of foot mycosis can be local but essential systemic treatment is required depending to the type and location of lesions. Currently the treatment of dermatophyte infections is usually long term, with several cases of recurrence [13].

Furthermore, systemic antifungal agents present many disadvantages such as therapeutic limitations with high toxicity, many drugs interactions and resistance [14]. In another hand, the study of antifungal susceptibility mechanism constitutes an important strategy to restrict the emergence of resistance to the commercially available agents and may help to provide the efficacy of an antifungal drug, so that the development of new and potential compounds is necessary [15].

Dermatophytes and NDMs have the ability to adhere on biotic or abiotic surface forming biofilms [16-18]. Fungal biofilm represent an important role in the pathogenesis and in the resistance to the antimicrobial agents[18]. Many methods have been developed to evaluate the quantity of biofilm [19,20]. The Cristal violet assay has been demonstrated to be the most reliable test, which stains metabolically active and inactive cells in mature biofilms [21].

The aims of the present study were to evaluate the *in vitro* ability to form biofilms and antifungal susceptibility of clinical isolates of dermatophytes and NDMs implicated in foot mycosis. Crystal Violet and Safranin Red staining quantified the amount of biofilm.

Materials and methods

Fungal strains

Clinical strains were isolated from patients with foot mycosis attending the Mycology Unit of the dermatology Department in the University Hospital la Rabta (Tunis) until a prospective study enrolled in Tunisia [22]. Thirty six strains were included in this study (Table 1), 26 dermatophyte species including *Trichophyton rubrum* (n=21), *Trichophyton interdigitale* (n=5), and 10 molds including *Scopulariopsis brevicaulis* (n=3), *Fusarium solani* (n=2), *Fusarium oxysporum* (n=2), *Chrysosporium keratinophylum* (n=2) and *Aspergillus terreus* (n=1). *T. mentagrophytes* DSM

4870 and *A. terreus* DSM 1958 from German Collection of Microorganisms (DSMZ, Braunschweig, Germany), were used as references strains. Initially, all isolates were identified using standard methods based on macroscopic and microscopic characteristics. Then, identification of fungal strains was confirmed by analysis of protein using MALDI-TOF MS (Parasitology-Mycology Unit in the Department of Microbiology, Necker-Enfants Malades Hospital AP-HP, Paris, France). Isolates were stored at – 80°C on Sabouraud Broth (Sigma Aldrich, St. Louis Missouri, USA) with 30% glycerol until the time of use.

Antifungal susceptibility assay

The minimal inhibitory concentration (MIC) on planktonic cells was determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute reference for filamentous fungi [23]. All strains were grown on Potato Dextrose Agar (Sigma Aldrich, St. Louis Missouri, USA) at 28-30°C until conidia formation. Inoculum suspension was prepared at final concentration of 0.4×10^4 to 5×10^4 CFU ml^{-1}. The *in vitro* antifungal activity was evaluated using five antifungal agents: fluconazole (FLC), econazole (ECO) (Sigma Aldrich, St. Louis Missouri, USA), with concentrations ranged from 64 to 0.125 µg ml^{-1} respectively and for itraconazole (ITC), terbinafine (TRB) and griseofulvin (GSF) (Sigma Aldrich, St. Louis Missouri, USA) from 16 to 0.032 µg ml^{-1}. MIC_{50} was defined as the lowest concentration that caused ≥ 50% growth inhibition; MIC_{80} was the lowest concentration that caused 80% growth inhibition and the MIC_{100} the lowest drug concentration that inhibited 100% of growth.

Evaluation of biofilm formation

The biofilm assay was performed with the use of methods described previously[19,24]. All strains were grown on Potato dextrose agar (Sigma Aldrich, St. Louis Missouri, USA), incubated at 28°C until sporulation. The inoculum was standardized to 1×10^6 conidia ml^{-1} in RPMI 1640 medium supplemented with L-glutamine, buffered with MOPS acid (Sigma–Aldrich), and added to 24-well plates. After 24-72h at 37°C, the cells were washed two times with sterile saline water 0.9% for removal of non-adherent cells. Morphology of biofilm was observed by light microscopy.

Biofilm quantification

Crystal Violet [19,24] and Safranin Red [25] bind to negatively charged molecules and can be used to stain and quantify total biomass comprising fungi and EPS Total Biomass

Cristal violet staining

After biofilm formation, the plates are dried at room temperature for 10 min and 1ml of 0.5% Crystal Violet solution was added to each well for 30 min. The wells were washed two times with sterile water to remove excess of crystal violet and biofilm were decolorized by the addition of 1ml of 80:20 ethanol/acetone solutions to each well. This solution was gently homogenized with a pipette until the rest of the crystal violet was completely dissolved (~ 1 min). Finally, the solution from each well was transferred to a new 96-well plate and then read in a microplate reader at 570 nm. All experiments were performed in triplicate.

Safranin staining

After the biofilm formation for 72h in 96-well plates, the plates were washed three times with 200µl of phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis Missouri, USA) and dried at 50°C for 30 min. Each well was stained with 50 µl of safranin solution 1% for 5 min, and then washed three times with 200 µl of PBS until the supernatant stayed clear. Finally, the optical density OD was read at 492 nm.

Antifungal susceptibility of dermatophyte and NDM biofilms

All strains were grown on Potato dextrose agar (Sigma Aldrich, St. Louis Missouri, USA), incubated at 28°C until sporulation. The inoculum was standardized to 1×10^6conidia ml^{-1} in RPMI 1640 medium supplemented with L-glutamine and buffered with MOPS acid (Sigma–Aldrich) and was allowed to form biofilm in 96-well polystyrene plates in the presence of five concentrations of ECZ and TER (16, 8, 4, 2 and 1 µg ml^{-1}). After incubation, the biofilm was quantified using crystal violet staining as previously described [20,23]. The concentrations causing 50% inhibition of biofilm formation due to drug treatment have been determinated.

Statistical analysis

The antifungal activities are the result of three independent experiments performed in duplicate. The data of antifungal activity (MIC) have been presented as median. In order to relate the biofilm content and, the MIC values of the different strains Pearson's correlation coefficient (r)

have been estimated. The correlation coefficient close to 1 indicates that the variables are positively and linearly related. The zero value indicates weak relationship between the variables, a correlation less than 0.5 is weak.

Results

The results of antifungal activity (MIC_{50}, MIC_{80}, and MIC_{100}) of FLC, ECO, ITC, TRB and GSF against planktonic cells of dermatophytes (*T. rubrum*, *T. interdigitale*) and NDMs (*F. solani*, *F. oxysporum*, *A. terreus*, *S. brevicaulis* and *C. keratinophylum* have been reported in Table 2. *Fusarium* spp., *S. brevicaulis* and A. *terreus* were resistant to FLC (MIC_{50} >64 µg ml^{-1}), ITC (MIC_{50} >16 µg ml^{-1}) and GSF (MIC_{80} >16 µg ml^{-1}). The strains of *F. solani* were resistant to all antifungal tested (MIC_{100}>16 µg $ml^{-1}$for ITC, TER, GSF and MIC_{50}>64 µg $ml^{-1}$for FLC, ECO). All the *Trichophyton* spp. strains were susceptible to TRB with MIC_{80} values of < 0.032 µg ml^{-1}. *Trichophyton spp*, causing tinea pedis showed resistance to FLC with MIC_{80} values of $\geq$ 64µg ml^{-1}.

All the strains demonstrated the ability to form biofilm on 24-well microtitration plate surface, however differences were observed among them (Fig. 1). *T. rubrum* (T21; T40 and T25) biofilms, *T.interdigitale* (T1) biofilm, *F. oxysporum* (M1 and M2) biofilms, *S. brevicaulis* (M3; M6 and M12) biofilms, *C. keratinophylum* (T37) biofilm and *A. terreus* (M5) biofilm produce a high amount of biomass (Fig. 1). In the present study, the species of *F. solani* (M7, M8) and *S. brevicaulis* (M3, M6) resistant clinical isolates, showed the most capacity to form biofilm on a polystyrene surface.

The results of biomass quantification of all isolates were presented in Figure 1, showed a correlation between Crystal violet (Fig. 1a) and Safranin staining amount (r = 0.694)(Fig 1b).

The amount of mature biofilm was not correlated to antifungal activity against all planktonic cells tested FLC-MIC_{80} values (r = 0.4422), FLC-MIC_{100} (r= 0.0584), ECO-MIC_{80} values (r=0.2135), ITC-MIC_{80} values (r=0.3534) and TRB-MIC_{80} values (r=0.1317).

The antifungal activity of ECO and TRB against biofilm formation was measured in terms of percentage of inhibition and the results are shown in Figure 2. The effect of antifungal agents against biofilm formation was measured in terms of the percentage of inhibition; the *in vitro* assay showed that at concentration of 4µg ml^{-1}, the percentage ranged from 0% to 95% for ECO

and from 4% to 93% for TRB. The best results were obtained with the ECO when compared with TRB on the strains tested.

However, for *Fusarium* species (M2 and M8), the in vitro biofilms assay showed a low susceptibility to the tested antifungal agents.

Discussion

The commonest agents of foot mycosis are dermatophytes such as the anthropophilic *T. rubrum* [26,27] also NDMSs like *Fusarium* spp., *S. brevicaulis* and *Aspergillus* sp. can be incriminated but in low rates [22,28-30]. The first step of treatment of tinea pedis and tinea unguium is to make precise diagnosis in order to provide the appropriate antifungal agent. Recently, the therapy of foot mycosis represents a major challenge, frequent failures and recurrent infection are observed [31-33], inappropriate selection of antifungal agents in addition to inadequate dose and duration of therapy could facilitates the rapid recurrence of infection and also the development of drug resistance**.** The methods of the *in vitro* antifungal activity can be useful to predict the capacity of a determined antifungal agent to detect the resistance trends and to eradicate the determined fungal species. To our knowledge, few studies have been conducted in Tunisia focused on the antifungal susceptibility among dermatophytes and NDMs responsible for foot mycosis. Actually, ITC, FLC and TRB are the most widely available antifungal agents used for systemic treatment of onychomycosis. In order to have successful therapy for biofilm onychomycosis, it is necessary to use an antifungal especially for biofilm degradation. Although, many systemic antifungal drugs had also been associated with some adverse side effects such as headache, hepatotoxicity, gastrointestinal disturbance (nausea, diarrhea, vomiting), skin rash and impotency [34,35] and for this reason it is important that therapy be preceded by drug sensitivity tests .

In this study, low MICs of ECO, ITC, TRB and GSF have been reported. However, FLC had the highest MIC value against all the clinical dermatophyte strains [36,37]. TRB was the most effective antifungal against *T.rubrum* and *T.interdigitale* species causing onychomycosis and tinea pedis. Previous studies [38-41] reported that TRB has a higher clinical cure with a slower relapse rates in a short period of treatment [42]. GSF was the first systemic treatment for skin and nail infections but demonstrate a limited spectrum activity to dermatophytes[43,44]. Moreover, GSF demonstrate a MIC values >16 µg ml^{-1} against some resistant dermatophyte isolates [44,45] In addition, we note

that GSF is less active against *T.rubrum* and *T. interdigitale* than the other antifungal agents beyond FLC.

Concerning the derivative azoles, ITC and ECO were demonstrated to be the most active agents against *Trichophyton* spp. agents of tinea pedis and tinea unguium[46,47]; otherwise, ITC was more effective in tinea pedis than ECO this finding is confirmed by study of Decroix. 1995 [48] showing a successful oral treatment of tinea pedis with ITC. However FLC showed a high MIC values (>64µg ml^{-1}) especially for species related to tinea pedis. These differences in the susceptibility can be explained by the fact that derivate azoles target fungal ergosterol in the structure differs among species.

In the present study, also NDMs such as *Fusarium* spp, *S. brevicaulis, and A. terreus* and *C. keratinophylum* were isolated from patients with foot mycosis. The treatment of tinea pedis and tinea unguium caused by NDMs is still not well standardized and many authors point out the poor therapeutic response of these fungal infections to systemic antifungal drugs [49-52]. We found that azoles (FLC, ECO, and ITC) and GSF showed a very high MICs values for the strains of *S. brevicaulis* and *Fusarium* spp. However, TRB presents a low MICs values for the strains of *S. brevicaulis* and *F. oxysporum*. In the other hand, the isolates of *F. solani* are resistant to all the antifungal tested, this can be explained by the characteristics of *Fusarium* species to be refractory and represents *in vivo* and *in vitro* resistance to most antifungal drugs [53-56].

The capacity of dermatophyte and NDM isolates to form biofilms is generally related to the ability to cause infection. In the present work, we have assessed the biofilm production by dermatophytes and NDMs associated to foot mycosis. Otherwise, a first work has reported the *in vitro* biofilm forming abilities of *T. rubrum* and *T. mentagrophytes* [19], and many other studies reported the biofilm formation of some filamentous fungi [25,57] . However, to our knowledge, the biofilm production by molds *S. brevicaulis* and *C. keratinophylum* associated to foot mycosis has not been described. Overall, all the isolates had the ability to adhere to the polystyrene surface and form biofilm in different degrees depending on the species. In the present study, the species of *F. solani* (M7, M8) and *S. brevicaulis* (M3, M6) resistant clinical isolates, showed the most capacity to form biofilm on a polystyrene surface. These finding let us suppose that the high production of biofilm, that is a permeability barrier surprisingly resistant to injury, could contribute to their survival, act as a persistent source of infection and further dissemination and account for antifungal resistance in onychomycosis [58,59] . The low susceptibility of *Fusarium*

biofilm (M2 and M8) to the tested antifungal agents, could confirmed the hypothesis of Seidler et *al.* [25].

The maturation of biofilm and the high cell density in the biofilm matrix may influence the different susceptibility to antifungal drugs.

Many factors suggest that biofilm represent an important role in the pathogenesis of onychomycosis including firm adherence of dermatophytes in the nail plate and ability to form biofilm, which increased of virulence and resistance to the antimicrobial agents (Nusbaum et al., 2012).

The differences in anti-biofilm assays, among dermatophyte and NDM species, can be related to the life cycle of biofilm especially in the maturation stage associated to the composition of the biofilm matrix and the rate of the drug diffusion through the biofilm.

Successful treatment of onychomycosis can be explained in first by the biofilm formation and for the susceptibility assay, antifungal agents should be tested among biofilms and not planktonic cells. Biofilm assays performed in vitro could allow for rapid screening of antifungal compounds. Appropriate selection of antifungal agents with adequate dose could help to resolve the infection and reduce its spread. In the recommendations given by ESCMID there is the urgent need to standardized biofilm susceptibility test and to biofilm-specific breakpoints for systemic and topically administered antibiotics [60].

Therefore, the increased levels of biofilm resistance underline the importance of developing assays to test biofilm antifungal susceptibilities. Such future research in antifungal drugs and their exact mode of action against dermatophyte and NDM biofilms are needed to be developed in order to target sessile cells.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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Figure legends

Figure 1. Quantification of biofilm biomass formation after 72h is represented by crystal violet absorbance at 570 nm (A) and safranin absorbance at 492 nm (B). Error bars represent standard deviation

Figure 2. Inhibition percentage of biofilm formation of dermatophyte and non-dermatophyte strains using different concentrations of econazole and terbinafine. Data are percentage of the mean of triplicates with respect to control. Error bars represent standard deviation.

Table1. Identification of dermatophyte and non-dermatophyte clinical strains used in this study

Strains	**Sex**	**Clinical diagnosis**	**Clinical aspect**	**Identification** (MALDI-TOF MS)
T4	F	*Tinea pedis*	PD	*T.rubrum*
T5	M	Onychomycosis	DLSO	*T.rubrum*
T7	M	Onychomycosis	DLSO	*T.rubrum*
T9	M	Onychomycosis	PSO	*T.rubrum*
T11	M	Onychomycosis	DLSO	*T.rubrum*
T12	F	Onychomycosis	TDO	*T.rubrum*
T13	F	Onychomycosis	DLSO	*T.rubrum*
T18	F	Onychomycosis	DLSO	*T.rubrum*
T21	F	Onychomycosis	DLSO	*T.rubrum*
T22 p	M	*Tinea pedis*	PH	*T.rubrum*
T23	F	Onychomycosis	TDO	*T.rubrum*
T25	F	Onychomycosis	DLSO	*T.rubrum*
T39	M	Onychomycosis	TDO	*T.rubrum*
T40	*M*	*Tinea pedis*	PH	*T.rubrum*
T42	M	Onychomycosis	PSO	*T.rubrum*
T46	F	Onychomycosis	DLSO	*T.rubrum*
T52	M	Onychomycosis	TDO	*T.rubrum*
T56	M	Onychomycosis	DLSO	*T.rubrum*
T61	F	*Tinea pedis*	ID	*T.rubrum*
T64	M	Onychomycosis	PSO	*T.rubrum*
T66	F	Onychomycosis	DLSO	*T.rubrum*

T103	M	Onychomycosis	DLSO	*T.rubrum*
T1	F	Onychomycosis	DLSO	*T.rubrum*
T34I	F	*Tinea pedis*	ID	*T.interdigitale*
T44	M	Onychomycosis	DLSO	*T.interdigitale*
T45	F	Onychomycosis	TDO	*T.interdigitale*
T68	F	Onychomycosis	TDO	*T.interdigitale*
M1	F	Onychomycosis	TDO	*F. oxysporum*
M2	F	Onychomycosis	DLSO	*F. oxysporum*
M3	M	Onychomycosis	DLSO	*S. brevicaulis*
M6	F	Onychomycosis	DLSO	*S. brevicaulis*
M12	F	Onychomycosis	DLSO	*S.brevicaulis*
M5	F	Onychomycosis	DLSO	*A. terreus*
M7	F	Onychomycosis	DLSO	*F. solani*
M8	F	Onychomycosis	DLSO	*F. solani*
T37	F	Onychomycosis	DLSO	*C. keratinophylum*
M13	M	Onychomycosis	DLSO	*C. keratinophylum*

NDMs: DLSO: Distal lateral subungual onychomycosis; ID: Interdigital; PD: Plantar dishydrosis; PH: Plantar hyperkeratosis; PSO: Proximal subungual onychomycosis; TDO: Total dystrophic onychomycosis. A: *Aspergillus*; C: *Chrysosporium*; F: *Fusarium*; S: *Scopulariopsis*; T: *Trichophyton*.

Table 2. Minimum inhibitory concentration (MIC) of five antifungal agents against dermatophyte and non-dermatophyte clinical strains

Antifungal agents	FLC MIC(μg ml^{-1})			ECO MIC(μg ml^{-1})			ITC MIC(μg ml^{-1})			TRB MIC(μg ml^{-1})			GSF MIC(μg ml^{-1})		
Strains	MIC_{50}	MIC_{80}	MIC_{100}	MIC_{50}	MIC_{80}	MIC_{100}	MIC_{50}	MIC_{80}	MIC_{100}	MIC_{50}	MIC_{80}	MIC_{100}	MIC_{50}	MIC_{80}	MIC_{100}
Dermatophytes (n=26)															
***Trichophyton rubrum* (n=21)**															
T4	32	64	>64	<0.125	<0.125	<0.125	ND	0.25	0.5	<0.031	<0.031	<0.031	0.5	1	ND
T5	4	16	64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.5	2	4
T7	16	16	>64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.5	1	ND
T9	32	64	>64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	4	8	>16
T11	1	1	16	<0.125	<0.125	<0.125	0.062	0.062	0.5	<0.031	<0.031	<0.031	>16	>16	>16
T12	0.125	0.125	4	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.5	1	2
T13	8	16	32	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	1	1	2
T18	32	32	64	<0.125	<0.125	<0.125	0.125	0.25	0.5	<0.031	<0.031	<0.031	1	2	4
T21	64	64	>64	<0.125	<0.125	<0.125	16	16	>16	<0.031	<0.031	<0.031	0.5	1	2
T22P	8	16	64	<0.125	<0.125	<0.125	0.125	0.125	0.25	<0.031	<0.031	<0.031	0.5	1	2
T23	0.5	1	2	<0.125	<0.125	<0.125	0.031	0.062	0.062	<0.031	<0.031	<0.031	1	2	4
T25	4	4	8	<0.125	<0.125	<0.125	0.125	0.25	0.5	<0.031	<0.031	<0.031	4	8	16
T39	4	8	32	<0.125	<0.125	<0.125	0.125	0.25	0.5	<0.031	<0.031	<0.031	ND	ND	ND
T40	16	64	>64	32	64	>64	0.25	0.25	0.5	<0.031	<0.031	<0.031	0.5	1	2

T42	8	32	>64	16	32	64	0.125	0.25	0.5	<0.031	<0.031	<0.031	0.5	1	>16
T46	16	16	>64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.5	1	ND
T52	>64	>64	>64	<0.125	<0.125	<0.125	0.125	0.125	0.5	<0.031	<0.031	<0.031	ND	ND	ND
T56	2	2	64	<0.125	<0.125	<0.125	0.065	0.065	0.065	<0.031	<0.031	<0.031	ND	ND	ND
T61	>64	>64	>64	<0.125	<0.125	<0.125	0.062	0.125	0.25	<0.031	<0.031	<0.031	1	2	4
T64	4	8	64	<0.125	<0.125	<0.125	0.062	0.25	0.5	<0.031	<0.031	<0.031	ND	ND	ND
T66	0.125	0.125	>64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	1	2	4
T103	0.5	2	64	<0.125	<0.125	<0.125	0.062	0.125	0.5	<0.031	<0.031	<0.031	ND	ND	ND
***Trichophyton interdigitale* (n=5)**															
T1	64	64	>64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.5	1	2
T34I	64	>64	>64	<0.125	<0.125	<0.125	0.125	0.25	0.25	<0.031	<0.031	<0.031	0.5	1	2
T44	0.5	0.5	1	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.5	1	>16
T45	64	64	>64	<0.125	<0.125	<0.125	0.065	0.065	0.125	<0.031	<0.031	<0.031	1	2	4
T68	>64	>64	>64	<0.125	<0.125	<0.125	0.125	0.25	0.5	<0.031	<0.031	<0.031	1	2	4
Molds (n=10)															
***Fusarium oxysporum* (n=2)**															
M1	>64	>64	>64	1	2	4	32	>16	>16	1	2	4	>16	>16	>16
M2	>64	>64	>64	4	8	16	>16	>16	>16	4	8	16	>16	>16	>16

***Fusarium solani* (n=2)**															
M7	>64	>64	>64	>64	>64	>64	>16	>16	>16	>16	>16	>16	>16	>16	>16
M8	>64	>64	>64	64	>64	>64	>16	>16	>16	>16	>16	>16	ND	ND	ND
***Scopulariopsis brevicaulis* (n=3)**															
M3	>64	>64	>64	16	>64	>64	>16	>16	>16	4	8	16	>16	>16	>16
M6	>64	>64	>64	8	16	>64	>16	>16	>16	1	2	8	>16	>16	>16
M12	>64	>64	>64	0.5	1	4	>16	>16	>16	0.25	0.25	2	>16	>16	>16
Aspergillus terreus															
M5	>64	>64	>64	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	>16	>16	>16
***Chrysosporium keratinophylum* (n=2)**															
T37	8	8	16	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	2	4	8
M13	2	4	8	<0.125	<0.125	<0.125	<0.031	<0.031	<0.031	<0.031	<0.031	<0.031	0.25	0.5	1
***T. mentagrophytes* DSM 4870**	64	64	>64	<0.031	<0.031	<0.031	>64	>64	>64	<0.031	<0.031	<0.031	1	2	4
***A. terreus* DSM 1958**	>64	>64	>64	<0.031	<0.031	<0.031	0.065	0.065	0.065	<0.031	<0.031	<0.031	>16	>16	>16

FLC: fluconazole; ECO: econazole; ITC: itraconazole; TRB: terbinafine; GSF: griseofulvin; MIC_{50} : the lowest concentration that caused ≥ 50% growth inhibition; MIC_{80} : the lowest concentration that caused 80% growth inhibition; MIC_{100} :the lowest drug concentration that inhibited 100% of growth.