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Suscetibilidade Antifúngica *in vitro* de Agentes de Feohifomicoses Superficiais

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RESUMO – A seleção de isolados fúngicos resistentes aos tratamentos disponíveis associada a um aumento no número de pacientes imunossuprimidos contribui para a incidência de infecções causadas por fungos demáceos. Assim, este estudo avaliou a eficácia terapêutica dos principais antifúngicos atualmente utilizados na prática clínica em relação à *Curvularia* spp. e *Hortaea werneckii* de casos de feohifomicose superficiais do sul do Brasil. O perfil de suscetibilidade para anfotericina B, fluconazol, itraconazol, terbinafina e voriconazol foi avaliado por microdiluição em caldo frente a fungos demáceos (*Curvularia lunata, C. pallescens* e *H. werneckii*). A terbinafina demonstrou maior eficácia contra *C. lunata* - média gemométrica (GM = 0,38 μg/mL), *C. pallescens* (MIC = 0,125 μg/mL) e *H. werneckii* (GM = 0,031 μg/mL) quando comparado aos demais antifúngicos testados. A maioria das espécies apresentou sensibilidade ao itraconazol e ao voriconazol, com uma concentração inibitória mínima (CIM) variando entre 1 - 8,0 μg/mL e 0,5 - 2,0 μg/mL, respectivamente. Todos os isolados testados apresentaram menor sensibilidade ao fluconazol (faixa de CIM 4 - 16 μg/mL). Embora o itraconazol seja considerado padrão ouro, a terbinafina demonstrou ser uma ótima alternativa para o tratamento das feohifomicose superficiais. O teste de suscetibilidade antifúngica é essencial para indicar a terapia ideal frente a essas infecções.
PALAVRAS-CHAVE – Antifúngicos; Brasil; Farmacorresistência Fúngica; Feoifomicose.

In vitro Antifungal Susceptibility of Agents for Superficial Phaeohyphomycosis

ABSTRACT – *The selection of fungal isolates resistant to available therapy associated with an increase in the number of immunosuppressed patients has contributed to the incidence of infections caused by dematiaceous fungi. Thus, this study evaluated the therapeutic efficacy of the main antifungal agents currently used in clinical practice in relation to Curvularia spp. and Hortaea werneckii from cases of superficial phaeohyphomycosis from southern Brazil. The susceptibility profile of amphotericin B, fluconazole, itraconazole, terbinafine and voriconazole against dematiaceous fungi (Curvularia lunata, C. pallescens and H. werneckii) was evaluated by microdilution in broth. Terbinafine showed greater efficacy against C. lunata - gemometric mean (GM = 0.38 μg/mL), C. pallescens (MIC = 0.125 μg/mL) and H. werneckii (GM = 0.031 μg/mL) when compared to the other antifungals tested. Most of species showed sensitivity to itraconazole and voriconazole, with a minimum inhibitory concentration (MIC) range from 1 - 8.0 μg/mL and 0.5 - 2.0 μg/mL, respectively. All isolates tested show low sensitivity to fluconazole (MIC range 4 - 16 μg/mL). Although itraconazole is considered gold standard, terbinafine has been showed to be a good alternative for the treatment of superficial phaeohyphomycosis. Lastly, antifungal susceptibility testing is essential to indicate the ideal therapy against these infections.*
KEYWORDS – *Antifungal Agents; Brazil; Drug Resistance, Fungal; Phaeohyphomycosis.*

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INTRODUCTION

Dematiaceous filamentous fungi are generally found in humid environments, such as decaying vegetation, bird nests, wood and soil, covering regions of tropical and subtropical climates.[1] These organisms are morphologically characterized by the formation of dark colonies, septate hyphae or black-yeasts elements.[2] The pigmentation of dematiaceous fungi is associated with the presence of melanin in its cell wall, which acts as an important virulence factor in the development of fungal infections in humans, such as phaeohyphomycosis.[3]

Ungual phaeohyphomycosis are examples of non-dermatophytic onychomycosis, responsible for 1.5% to 18% of the cases of these infections.[4,5] Phaeohyphomycosis have increased their incidence in humans, both in immunocompromised and immunocompetent individuals,[6] and cause nail disease presenting as changes in color, thickness, detachment and onychodystrophy.[7]

The genus *Curvularia* has a worldwide coverage that is mostly found in soil and in plants.[2] Due to this, most cases of phaeohyphomycosis induced by these fungi occur in men aged 30-50 years, working in rural areas or other occupations that expose them to plant materials.[8,9] *H. werneckii*, although with worldwide distribution, is mainly found in regions of tropical and subtropical climates of Central and South America, Africa and Asia and in places with high salt concentration.[10,11] This species has peculiar structural characteristics, because its yeast and filamentous forms coexist. Initially, colonies are restricted, black, and similar to yeast (yeast-like fungi)[12] and over time, fungal structures become broad septate and dark hyphae, mostly with a filamentous appearance.[13] *Tinea nigra* is a superficial phaeohyphomycosis caused by *H. werneckii*,[14] which presents as brown to black macules with sharp margins and irregular shapes, usually located on the palms and soles.[11]

Treatment of dematiaceous infections can be performed with different classes of antifungals.[13] Azoles like itraconazole (ITC), voriconazole (VRC) and posaconazole (PSC) are considered the first choice and most effective drugs.[1,13,15] According to Wong & Revankar,[16] generally amphotericin B (AMB) has satisfactory activity against most of the dematiaceous fungi and some studies have shown that terbinafine (TRB) is highly effective against these infections.[14,17] On the other hand, fluconazole (FLC) and ketoconazole (KTC) are poorly active against phaeohyphomycosis.[1,16] Specifically for the genus *Curvularia*, the treatment of choice is not yet fully established. According to Aguas *et al*[18] the use of AMB, miconazole (MCZ), CTC, TRB and ITC present satisfactory results. In parallel, studies demonstrate that therapy with ITC and TRB has been effective in cases of *Tinea nigra* caused by *H. werneckii*.[19,20]

Currently, the selection of fungal isolates resistant to available antifungals has contributed to the increase of infections caused by dematiaceous fungi.[21] However, reports involving the susceptibility profile of these fungi are scarce. Thus, the objective of this study was to evaluate the *in vitro* activity of the main antifungal agents against *Curvularia* spp. and *H. werneckii* isolates cultured from cases of superficial phaeohyphomycosis.

MATERIAL AND METHODS

Fungal isolates

A total of nine isolates of dematiaceous fungi were used in this study: *C. lunata* (CLU 01, CLU 02, CLU 03, CLU 04 e CLU 05), *C. pallescens* (CPA 01) e *Hortaea werneckii* (HWE 01, HWE 02 e HWE 03). The organisms are deposited in the collection of cultures of Laboratório de Micologia Aplicada, Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil) and were used in this study. All isolates were stored on potato dextrose agar (PDA) and kept at room temperature for the period necessary to perform all the tests. *Candida albicans* ATCC 18804 strain was obtained from American type culture collection (ATCC) and used as a strain control for the test. The fungal isolates were confirmed according to the taxonomic keys.[22-24]

Antifungals agents

Amphotericin B (AMB) (Anforicin B® - Cristália, São Paulo, Brazil), fluconazole (FLC) (Sigma, St. Louis, MO), itraconazole (ITC) (Janssen-Cilag Farmacêutica® - São Paulo, Brazil), terbinafine (TRB) (Funtyl® - Cristália, São Paulo, Brazil) and voriconazole (VRC) (Vfend® - Pfizer, Nova York, NY) were used in this study. The antifungal solutions were prepared in RPMI broth1640 (Sigma, St. Louis, MO) at128 μg/mL (AMB, FLC e ITC) and 16 μg/mL (VRC e TRB) according to Clinical and Laboratory Standards Institute (CLSI, 2008). Stock solutions were prepared and stored at - 20°C until use.

Antifungal susceptibility testing

Curvularia spp. and *H. werneckii* isolates were cultivated in Potato Dextrose Agar (Kasvi, Paraná, Brazil) and incubated at 32°C for 5-7 days. Then, suspensions containing these isolates were prepared in a sterile saline solution 0.85%. The concentration of each fungal suspension was determined in a spectrophotometer (GT7220, Global Trade Technology) at 530 nm. The optical density varied between 0.09 – 0.13 for *H. werneckii* and 0.25 – 0.3 for *Curvularia* spp., obtaining approximately 0.4 a 5 x 104 CFU/mL further used for testing. Antifungal solutions were prepared in RPMI 1640 culture medium at128 μg/mL (AMB, FLC and ITC) and 16 μg/mL (VRC and TRB), obtaining a concentration range between 128 - 0.125 μg/mL and 16 - 0.031 μg/mL, respectively. The microplates containing the antifungal solutions and each fungal suspension were incubated at 32°C for 2 and 5 days, for *Curvularia* spp. and *H. werneckii*, respectively. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of antifungal capable of inhibiting 50% (FLC and ITC), 80% (TRB) and 100% (AMB and VRC) of fungal growth when compared to each isolate without treatment. The methodology followed the proposed by document M38 – A2.[25] For the ATCC 18804 strain, the assay followed the protocol M27 - A3,[26] with interpretation of the breakpoints according to protocol M27-S4.[27]

Tabe 1 - Minimum inhibitory concentration (MIC) values of antifungal agents against *Curvularia* spp. and *Hortaea werneckii* isolates.

Isolates	Antifungal	MIC range (μg/mL)	Geometric mean (μg/mL)
Curvularia lunata (n = 5)	AMB	0.5 – 4.0	1.52
	FLC	4.0 – 16.0	10.56
	ITC	1.0 – 8.0	2.64
	TRB	0.125 – 1.0	0.38
	VRC	0.5 – 2.0	1.15
Curvularia pallescens (n = 1)	AMB	0,5	-
	FLC	8.0	-
	ITC	4.0	-
	TRB	0.125	-
	VRC	0.5	-
Hortaea werneckii (n = 3)	AMB	16 - > 64	32.0
	FLC	8.0 – 8.0	8.0
	ITC	1.0 – 4.0	2.0
	TRB	0.015 – 0.062	0.031
	VRC	0.25 - > 8.0	1.26

AMB: amphotericin B; FLC: fluconazole; ITC: itraconazole; TRB: terbinafine; VRC: voriconazole.

RESULTS

The isolates from superficial phaeohyphomycosis were confirmed for the species from Laboratório de Micologia Aplicada (Porto Alegre, Brazil): *C. lunata* (n = 5), *C. pallescens* (n = 1) e *H. werneckii* (n = 3). *C. lunata* isolates presented conidia containing three septa, simple septa, ellipsoids and light brown cells with hyaline at their ends. *C. pallescens* presented conidial isolates, containing three to four septa in their majority, ellipsoids to fusiformes, sub-hyaline to light brown coloration, with the third cell disproportionally larger. *H. werneckii* isolates showed large, septate and dark hyphae with conidia of ellipsoidal aspect, brown and with double dark central septa.

Different MIC were obtained for the drugs tested. TRB demonstrated greater efficacy against *C. lunata* (Geometric mean (GM) = 0.38 μg/mL) and *H. werneckii* (GM = 0.031 μg/mL), when compared to the other antifungals. ITC was active against all genus evaluated (MIC range 1 to 8 μg/mL). VRC and AMB presented satisfactory activity against the *Curvularia* spp. species. However, for two *H. werneckii* isolates, these drugs were not very effective: VRC (MIC = 8 μg/mL) and AMB (MIC > 64 μg/mL). For FLC, all isolates tested showed low sensitivity (MIC range 8 to 16 μg/mL).

For *Curvularia* genus, the antifungal susceptibility was similar for all isolates. For *C. lunata*, FLC was the least active antifungal (GM = 10.56 μg/mL), followed by ITC (GM = 2.64 μg/mL), AMB (GM = 1.52 μg/mL), VRC (GM = 1.15 μg/mL) and TRB was the most effective drug (GM = 0.38 μg/mL). For *C. pallescens*, FLC showed lower efficacy (MIC = 8 μg/mL), followed by ITC (MIC = 4 μg/mL), AMB and VRC (MIC = 0.5 μg/mL) and TRB (MIC = 0.125 μg/mL). In contrast, AMB (GM = 32 μg/mL) was the least effective drug against *H. Werneckii* isolates, followed by FLC (GM = 8 μg/mL), ITC (GM = 2 μg/mL), VRC (GM = 1.26 μg/mL) and TRB (GM = 0.031 μg/mL) (Table 1).

DISCUSSION

The interpretative criteria for evaluation of susceptibility of dematiaceous filamentous fungi to clinically available antifungals are not well established,[25] due to the lack of clinical breakpoints defined against these fungal species *in vitro*. Thus, fungal susceptibility testing is essential for the identification of resistant isolates and indication of appropriate treatment against superficial phaeohyphomycosis[28] which will depend on the severity and initial site of the infection, along with host factors such as chronic diseases, immunocompromising state and age.[29]

Most dematiaceous fungi isolates were not susceptible to FLC (MIC between 16 to 64 μg/mL), as previously reported.[4] Previous data showing MIC > 8 μg/mL suggests non-dermatophytic species are resistant for ITC.[30] According to Yew *et al*,[31] dematiaceous fungi present high susceptibility to AMB, ITC and VRC (MIC ≤ 1 μg/mL), however, low sensitivity to FLC (MIC ≥ 4 μg/mL).

Curvularia species showed low sensitivity to ITC and VRC but previous reports have shown a higher efficacy to these drugs (MIC range 0.004 - 2 μg/mL),[7,13,15,32,33] also contrasting with for Da Cunha *et al*[13] who showed low efficacy ITC and VRC when evaluated against some *Curvularia* species. Recent studies showed that AMB has been a good alternative

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for phaeohyphomycosis (MIC < 2 μg/mL),[16,28,34,35] since *Curvularia* spp. isolates presented satisfactory sensitivity to this drug, but there are contradictory results.[13]

Nizam *et al*[33] and Krizsán *et al*[35] described MIC > 8 μg/mL for TRB against the genus *Curvularia*, but in contrast, our results demonstrated that TRB was highly effective against all isolates of this genus.

ITC and VRC were effective for *H. werneckii* isolates. However, VRC had elevated MIC values to some isolates, whereas MIC > 8 μg/mL had not previously been shown for VRC against this specie.[36,37] All *H. werneckii* isolates showed low sensitivity to AMB, in agreement with studies by Formoso *et al*.[37] Finally, TRB was highly effective against all *H. werneckii* isolates.

CONCLUSION

Although there was no definitive correlation between the in vitro susceptibility and the clinical response to the antifungals, this study may be importance to elucidate the susceptibility profile of antifungals available for the treatment of superficial phaeohyphomycosis. *Curvularia* spp. isolates showed greater sensitivity to available therapy when compared to *H. werneckii* isolates. Although ITC is considered the first choice, TRB has been shown to be an excellent option for the treatment of phaeohyphomycosis. Thus, the evaluation of the efficacy of antifungal agents is essential to indicate the ideal therapy against these infections.

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