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Short Communication

In vitro activity of nine antifungal agents against a global collection of *Hortaea werneckii* isolates, the agent of tinea nigra

Hamid Badali[a,b,*], Abdullah M.S. Al-Hatmi[c,d,e], Hamed Fakhim[f,g], Aidan Moghaddasi[a,h], Sadegh Khodavaisy[i,j], Afsane Vaezi[a,h], Fatemeh Ahangarkani[b,h], G. Sybren de Hoog[c,e], Jacques F. Meis[e,k]

[a] *Department of Medical Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
[b] *Invasive Fungi Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
[c] *Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands*
[d] *Ministry of Health, Directorate General of Health Services, Ibri, Oman*
[e] *Centre of Expertise in Mycology, Radboud University Medical Centre/Canisius Wilhelmina Hospital, Nijmegen, the Netherlands*
[f] *Department of Medical Parasitology and Mycology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran*
[g] *Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran*
[h] *Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran*
[i] *Zoonoses Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran*
[j] *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[k] *Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, the Netherlands*

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ABSTRACT

The in vitro susceptibility of molecularly identified *Hortaea werneckii* isolates ($n = 37$), the causative agent of tinea nigra, originating from clinical and environmental sources was determined for nine antifungal agents. Posaconazole had the lowest geometric mean minimum inhibitory concentration (GM MIC) (0.07 µg/mL), followed by voriconazole (0.13 µg/mL), isavuconazole (0.14 µg/mL), itraconazole (0.16 µg/mL), terbinafine (0.19 µg/mL) and amphotericin B (0.92 µg/mL). In contrast, fluconazole (14.56 µg/mL), caspofungin (2.41 µg/mL) and anidulafungin (1.42 µg/mL) demonstrated the highest GM MICs/MECs against *H. werneckii*.

1. Introduction

The hyphomycete genus *Hortaea* represents anamorph members of the ascomycetes in the order Capnodiales comprising the black yeast-like fungi [1]. The genus was established in 1984 and at present consists of three species, namely *Hortaea werneckii* (type species), *Hortaea acidophila* and *Hortaea thailandica* [1–3]. *Hortaea werneckii* (Horta) was previously classified as *Cladosporium werneckii* (Horta), *Exophiala werneckii* (Horta) Arx, and *Phaeoannellomyces werneckii* (Horta). It is a halotolerant fungus occurring mainly in coastal areas in (sub)tropical climates, where it resides in shallow water or man-made salt pans [4,5]. In rare instances it has been recovered from house dust [6]. *Hortaea werneckii* is the aetiological agent of tinea nigra, a superficial mycosis with scaling and brown or black macules affecting the stratum corneum of the palms and soles [7]. Spread of the infection to other sites is rare, pigmentation is irregularly distributed, and lesions often remain asymptomatic [7]. Generally, tinea nigra is considered to be a tropical disease, prevalent in warm, humid parts of the world, i.e. Central America, South America, Africa and Asia, and it is also infrequently encountered in Australia and the southern USA [8–11]. In contrast, it is considered an imported fungal infection in Europe [12]. Owing to the high degree of phenotypic similarity between closely related isolates, identification problems are well known and *H. werneckii* is generally misidentified. Moreover, isolates show differences in susceptibility to antifungal agents [7,13]. Medical therapy is based on experience reported in case reports mostly involving Whitfield's ointment, miconazole, ketoconazole, itraconazole, thiabendazole, bifonazole and terbinafine [7,14–18]. Alternative antifungal drug regimens with fewer side effects may help to manage infections [18,19]. The purpose of the present

* Corresponding author. Department of Medical Mycology, Invasive Fungi Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. Tel.: +98 91 2841 3720.
E-mail address: badalii@yahoo.com (H. Badali).

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Table 1
Origin, source and MIC/MEC data of clinical and environmental *Hortaea werneckii* strains ($n = 37$)

Country	CBS number	GenBank ITS rDNA	Source	Origin	MIC (μg/mL)								
					AmB	FLU	ITR	VRC	POS	ISA	CFG	AFG	TER
Mexico	122342	**MH028899**	C	Tinea nigra	2	16	0.25	0.125	0.125	0.25	4	2	0.25
	122344	**MH028900**	C	Tinea nigra	2	16	0.25	0.125	0.125	0.25	2	2	0.25
	123041	**MH028901**	C	Tinea nigra	2	8	0.25	0.125	0.125	0.25	4	2	0.25
	123042	**MH028902**	C	Tinea nigra	2	16	0.25	0.125	0.063	0.25	2	1	0.25
	123043	**MH028903**	C	Tinea nigra	2	16	0.25	0.125	0.125	0.25	4	2	0.25
	123044	**MH028904**	C	Tinea nigra	2	16	0.25	0.25	0.125	0.25	4	2	0.25
	123045	**MH028905**	C	Tinea nigra	2	8	0.25	0.125	0.063	0.125	4	2	0.25
	123046	**MH028906**	C	Tinea nigra	2	16	0.25	0.25	0.125	0.25	2	2	0.25
	126984	**MH028907**	C	Tinea nigra	2	16	0.125	0.125	0.125	0.125	2	1	0.125
	126987	**MH028908**	C	Tinea nigra	2	16	0.25	0.125	0.063	0.25	2	1	0.25
	126985	**MH028909**	C	Tinea nigra	2	8	0.125	0.125	0.125	0.25	2	1	0.125
	126986	**MH028910**	C	Tinea nigra	2	8	0.25	0.125	0.063	0.125	2	1	0.25
	122348	**MH028911**	C	Tinea nigra	1	16	0.25	0.125	0.125	0.25	4	2	0.25
	122340	**MH028912**	C	Tinea nigra	2	16	0.5	0.25	0.125	0.25	1	1	0.125
Brazil	111.31	**AJ238679.1**	C	Tinea nigra	0.5	16	0.25	0.5	0.125	0.5	4	4	0.25
	117.90	**AJ238472.1**	E	Salted fish	1	16	0.125	0.125	0.063	0.125	2	1	0.125
	115.90	**AJ238470.1**	C	*Bufo granulosus*, kidney	0.25	32	0.25	0.25	0.125	0.25	4	4	0.25
	120937	**MH028954**	E	Plant leaf	0.125	16	0.25	0.25	0.063	0.25	2	1	0.25
Slovenia	100455	**AY128704.1**	E	Sea water	0.125	8	0.016	0.031	0.016	0.031	2	1	0.031
	100457	**MH028913**	E	Saline water	0.25	32	0.125	0.25	0.063	0.125	8	2	0.5
	100456	**MH028914**	E	Saline water	0.25	32	0.25	0.125	0.063	0.125	4	2	0.125
Spain	373.92	**AJ238474.1**	E	Beach soil	0.125	16	0.063	0.063	0.031	0.063	2	1	0.125
	117931	**MH028898**	E	Limestone rock	0.25	16	0.031	0.063	0.031	0.063	1	0.5	0.063
Suriname	359.66	**AJ244249.1**	C	Tinea nigra palmaris	2	16	0.125	0.125	0.031	0.063	4	0.5	0.125
Portugal	107.67 (T)	**AJ238468.1**	C	Tinea nigra	2	8	0.063	0.125	0.031	0.063	2	1	0.25
Senegal	706.76	**MH028955**	E	*Rhizophora mangle*, leaf	0.5	16	0.063	0.063	0.031	0.063	1	1	0.125
Sri Lanka	707.76	**MH028915**	E	Sooty mould	2	16	0.125	0.125	0.063	0.063	2	2	0.25
Netherlands	123850	**MH028916**	E	Salt bath for salting cheeses	1	8	0.25	0.25	0.125	0.25	4	4	0.25
Sudan	110352	**MH028917**	E	Hollow tree	1	8	0.125	0.125	0.063	0.125	2	2	0.25
Puerto Rico	120952	**MH028918**	E	Hypersaline water	2	64	0.25	0.25	0.125	0.25	8	4	0.5
Greece	100496	**AY128703.2**	E	Sea water-sprayed marble	1	8	0.125	0.125	0.063	0.125	2	1	0.25
Japan	410.51	**MH028919**	E	Air	1	16	0.125	0.125	0.063	0.125	2	1	0.125
France	705.76	**MH028920**	C	Tinea nigra	1	16	0.125	0.063	0.031	0.063	1	1	0.063
Italy	126.35	**MH028921**	C	Tinea nigra	2	32	0.25	0.25	0.125	0.25	1	2	0.25
Unknown	116.90	**AJ238471.1**	C	Cantharus, eye infection	0.25	8	0.125	0.125	0.063	0.125	4	2	0.5
	122.32	**AJ238473.1**	E	Unknown	1	16	0.5	0.25	0.125	0.25	1	1	0.25
	708.76	**MH028922**	C	Tinea nigra	0.5	8	0.25	0.125	0.063	0.063	2	0.5	0.125

MIC, minimum inhibitory concentration; MEC, minimum effective concentration; ITS, internal transcribed spacer; AmB, amphotericin B; FLU, fluconazole; ITR, itraconazole; VRC, voriconazole; POS, posaconazole; ISA, isavuconazole; CFG, caspofungin; AFG, anidulafungin; TER, terbinafine; C, clinical; E, environmental.

study was to determine the in vitro susceptibility of a collection of environmental and clinical *H. werneckii* isolates to nine antifungal agents, including the novel triazole isavuconazole.

2. Materials and methods

2.1. Isolates and identification

A panel of 37 well-characterised *H. werneckii* isolates, comprising 22 clinical and 15 environmental isolates from different geographical regions, was obtained from the reference collection of the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). Isolates were identified through sequencing of the internal transcribed spacer (ITS) rDNA region.

2.2. In vitro antifungal susceptibility testing

Because *H. werneckii* grows as a filamentous fungus, minimum inhibitory concentrations (MICs) and minimum effective concentrations (MECs; for echinocandins only) were determined according to Clinical and Laboratory Standards Institute (CLSI) document M38-A2 [20]. *Hortaea werneckii* is not a true yeast [21] and therefore M38-A2 was used because we aimed to read at 100% inhibition and not at 50% which, except for amphotericin B, is employed with true yeasts. Antifungal agents were dispensed into 96-well microtitre plates at final concentrations of 0.016–16 μg/mL for amphotericin B (Bristol-Myers-Squib, Woerden, the Netherlands), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central Research, Sandwich, UK), isavuconazole (Basilea Pharmaceuticals, Basel, Switzerland), posaconazole (Merck Sharp & Dohme, Haarlem, the Netherlands) and caspofungin (Merck Sharp & Dohme), 0.063–64 μg/mL for fluconazole (Pfizer Central Research), 0.08–8 μg/mL for anidulafungin (Pfizer Central Research) and 0.004–4 μg/mL for terbinafine (Novartis Research Institute, Vienna, Austria). All antifungal stock solutions were prepared in dimethyl sulfoxide (DMSO), with a final concentration of DMSO in the test wells of ≤1%. Briefly, all isolates were grown on potato dextrose agar plates at 35°C for up to 7 days to induce adequate sporulation, with the inoculum suspensions being prepared under appropriate biosafety methods by slightly scraping the surface of mature colonies with a loop and suspending the resulting material in sterile saline containing 0.05% Tween 40. If large aggregates were present they were allowed to settle for 5 min, then the homogeneous conidial suspension was transferred to sterile tubes and the supernatants were adjusted spectrophotometrically at 530 nm wavelength to an optical density ranging from 0.09 to 0.13. The final size of the stock inoculum ranged from 0.4×10^4 to 3.1×10^4 CFU/mL as determined by quantitative colony counts on Sabouraud

Table 2
In vitro susceptibilities of clinical and environmental isolates of *Hortaea werneckii* to nine antifungal agents

Source of isolation	Antifungal agent	MIC/MEC (μg/mL)			
		MIC range	MIC_{50}	MIC_{90}	GM MIC
All isolates ($n=37$)	Amphotericin B	0.125–2	1	2	0.92
	Fluconazole	8–64	16	32	14.56
	Itraconazole	0.016–0.5	0.25	0.25	0.16
	Voriconazole	0.031–0.5	0.125	0.25	0.13
	Posaconazole	0.016–0.125	0.063	0.125	0.07
	Isavuconazole	0.031–0.5	0.125	0.25	0.14
	Caspofungin[a]	1–8	2	4	2.41
	Anidulafungin[a]	0.5–4	1	4	1.42
	Terbinafine	0.031–0.5	0.25	0.25	0.19
Clinical isolates ($n=22$)	Amphotericin B	0.25–2	2	2	1.37
	Fluconazole	8–32	16	16	13.66
	Itraconazole	0.063–0.5	0.25	0.25	0.20
	Voriconazole	0.063–0.5	0.125	0.25	0.15
	Posaconazole	0.031–0.125	0.125	0.125	0.08
	Isavuconazole	0.063–0.5	0.25	0.25	0.17
	Caspofungin	1–4	2	4	2.49
	Anidulafungin	0.5–4	2	2	1.45
	Terbinafine	0.063–0.5	0.25	0.25	0.20
Environmental isolates ($n=15$)	Amphotericin B	0.125–2	1	2	0.52
	Fluconazole	8–64	16	32	16
	Itraconazole	0.016–0.5	0.125	0.25	0.11
	Voriconazole	0.031–0.25	0.125	0.25	0.12
	Posaconazole	0.016–0.125	0.063	0.125	0.05
	Isavuconazole	0.031–0.25	0.125	0.25	0.11
	Caspofungin	1–8	2	8	2.29
	Anidulafungin	0.5–4	1	4	1.38
	Terbinafine	0.031–0.5	0.25	0.4	0.17

MIC, minimum inhibitory concentration; MEC, minimum effective concentration; GM, geometric mean.
[a] For caspofungin and anidulafungin, MICs were read as MECs.

glucose agar (BD Difco, Vianen, the Netherlands). The inoculum suspensions, including mainly non-germinated conidia, were diluted 1:50 in RPMI 1640 medium. Microdilution plates were incubated at 35°C for 72 h (some plates with insufficient growth were incubated for 96 h). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were chosen as quality control strains to be used with every new series of MIC plates. The MIC endpoints of amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole were determined with the aid of a reading mirror as the lowest concentration of drug that inhibited recognisable growth (100% inhibition compared with the drug-free growth control), fluconazole as the level that induced a prominent reduction of growth (>50% inhibition) and terbinafine a 80% growth inhibition. MECs for caspofungin and anidulafungin were defined microscopically as the lowest concentrations of drug that led to the growth of small, rounded, compact hyphal forms compared with the long, unbranched hyphal clusters that were seen in the growth controls.

3. Results

Tables 1 and 2 summarise the distribution of *H. werneckii* isolates according to their geographic origin, source, MIC/MEC range, geometric mean (GM) MIC/MEC and MIC_{50}/MIC_{90}. The isolates originated from Mexico ($n=14$), Brazil ($n=4$), Slovenia ($n=3$), Spain ($n=2$), Suriname ($n=1$), Portugal ($n=1$), Senegal ($n=1$), Sri Lanka ($n=1$), the Netherlands ($n=1$), Sudan ($n=1$), Puerto Rico ($n=1$), Greece ($n=1$), Japan ($n=1$), France ($n=1$) and Italy ($n=1$), whereas 3 isolates had an unknown origin. Nucleotide sequence accession numbers of the isolates in GenBank are listed in Table 1. The MICs of isavuconazole ranged from 0.031–0.5 μg/mL, similar to posaconazole (0.016–0.125 μg/mL), itraconazole (0.016–0.5 μg/mL), voriconazole (0.031–0.5 μg/mL) and terbinafine (0.031–0.5 μg/mL). Amphotericin B (0.125–2 μg/mL), anidulafungin (0.5–4 μg/mL), caspofungin (1–8 μg/mL) and fluconazole (8–64 μg/mL) had higher MICs/MECs. Posaconazole had the lowest GM MIC (0.07 μg/mL), followed by voriconazole (0.13 μg/mL), isavuconazole (0.14 μg/mL), itraconazole (0.16 μg/mL), terbinafine (0.19 μg/mL), amphotericin B (0.92 μg/mL), anidulafungin (1.42 μg/mL), caspofungin (2.41 μg/mL) and fluconazole (14.56 μg/mL). Although isavuconazole had potent activity with MIC_{50} and MIC_{90} values of 0.125 μg/mL and 0.25 μg/mL, respectively, the MIC_{50} of isavuconazole was 1 $\log_2$ dilution step higher than that of posaconazole (0.063 μg/mL). Echinocandins had poor activity both against clinical and environmental isolates as expected for melanised fungi, with MIC_{90} values that were >4 $\log_2$ dilution steps higher than those of isavuconazole and posaconazole. Similarly, based on GM MICs, isavuconazole (0.14 μg/mL) was slightly more active than terbinafine (0.19 μg/mL). There were no significant differences in activities between environmental and clinical isolates ($P > 0.05$).

4. Discussion

Tinea nigra is an uncommon discoloration of the skin caused by *H. werneckii*. Most reports originate from tropical and humid climate zones. *Hortaea werneckii* is an outdoor melanised meristematic saprophyte and occasionally can also be present in house dust [1,6]. Studies have estimated that the frequency of tinea nigra may be higher, but due to its asymptomatic nature and the possibility of spontaneous cure, patients seldom seek medical attention [7,10]. In addition, tinea nigra has been misidentified as melanoma or pigmented naevi [12]. Successful treatment of tinea nigra has been reported using topical treatment with keratolytic agents or systemic treatment with ketoconazole, bifonazole or terbinafine and, more recently, with itraconazole and voriconazole [7]. *Hortaea werneckii* is halophilic, having the capacity to resist high salt concentrations, and therefore proper treatment should include control of hyperhidrosis, independent of the selected antifungal agent. *Hortaea werneckii* is not only involved in superficial

cases but in rare instances also with invasive disease for which the current results are more relevant. A retrospective report by Bonifaz et al. [7] showed that predisposing factors are associated with hyperhidrosis and exposure to hypersaline environments where the causative agent may be picked up from the natural habitat. Eleven cases with tinea nigra were treated with Whitfield ointment. Using the antifungals ketoconazole 2%, bifonazole 1% and terbinafine 1%, all patients achieved clinical and mycological cure within 12–18 days, with a mean total treatment period of 15 days [7]. Shadomy et al. reported that *H. werneckii* was inhibited in vitro by ketoconazole, econazole and oxiconazole [22]. The present study showed that all triazoles and terbinafine had potent activity against *H. werneckii*. Likewise, McGinnis and Pasarell demonstrated that itraconazole (0.03–0.25 μg/mL), voriconazole (0.03–0.125 μg/mL) and amphotericin B (0.03–1 μg/mL) had activity against 11 *H. werneckii* isolates [23], and Ng et al. showed that amphotericin B was not a good candidate for the treatment of invasive *H. werneckii* infection [24]. Remarkably, Brazilian environmental isolates had also high MICs for amphotericin B [13]. The latter study used only four environmental isolates and employed CLSI standard M27-A3, but surprisingly the MIC reading of all drugs was done at 100% inhibition, which is de facto the same as CLSI M38-A2 used in the current study. Topical isoconazole twice daily for 20 days was used in a 62-year-old male patient from Ethiopia with dark plaques on the palms of both hands, with complete resolution of the palmar lesions without relapse during 1 year of follow-up [25]. In the present study, fluconazole (GM MIC 14.56 μg/mL) had the lowest in vitro activity among the azoles, which contrasted with the data of Ng et al. [24]. High GM MECs were observed for anidulafungin (1.42 μg/mL) and caspofungin (2.41 μg/mL), which is a common observation for melanised fungi [26]. In conclusion, here we have compiled the first comprehensive study on antifungal susceptibility data of a large collection of *H. werneckii* isolates and the results suggest that azoles, including isavuconazole, were in vitro the most active drugs. Posaconazole appears to have a more favourable adverse effect profile than voriconazole [27], as has isavuconazole. Overall, in vitro susceptibility testing can help to select an appropriate therapy when managing patients with *H. werneckii* infection.

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Competing interests

JFM has received grants from Astellas, Basilea and Merck, has been a consultant to Astellas, Basilea, SCYNEXIS and Merck, and has received speaker's fees from Merck, United Medical, TEVA and Gilead Sciences. All other authors declare no competing interests.

Ethical approval

Not required.

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