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In vitro susceptibilities of 11 clinical isolates of *Exophiala* species to six antifungal drugs

In-vitro-Empfindlichkeit 11 klinischer Isolate von *Exophiala*-Arten für sechs Antimyzetika

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Summary. The antifungal activities of miconazole, terbinafine, itraconazole, UR 9825, voriconazole and amphotericin B against 11 clinical isolates of *Exophiala* spp. were tested by the broth microdilution method. All drugs were very active against *Exophiala* spp.. The 90% minimal inhibitory concentration (MIC_{90}) ranged from 0.125 to 1 μg ml^{-1}. Terbinafine was the most active drug against *Exophiala spinifera*, *Exophiala dermatitidis* and *Exophiala castellanii* and seems to be a promising agent in the treatment of infections caused by these fungi.

Zusammenfassung. Die antimyzetische Aktivität von Miconazol, Itraconazol, Voriconazol, UR 9825, Terbinafin und Amphotericin B gegen klinische *Exophiala*-Isolate wurde im Mikrodilutionstest geprüft. Alle Antimyzetika erwiesen sich als aktiv (MHK_{90} 0.125–1 μg/ml). Terbinafin war am wirksamsten gegen *Exophiala spinifera, Exophiala dermatitidis* und *Exophiala castellanii* und scheint für die Behandlung solcher Infektionen vielversprechend zu sein.

Introduction

Dematiaceous fungi are being increasingly recognized as human pathogens [1] particularly in immunocompromised patients [2]. Members of the genus *Exophiala* which belong to this group, are widely distributed in the environment, in soil, wood and other plant matter [3] and have been considered as agents of mycetoma, chromoblastomycosis and phaeohyphomycosis [4]. *Exophiala* spp. are most often recovered from subcutaneous nodules and skin lesion resulting from traumatic inoculation [5]. The infections caused by these species may be acquired through inhalation and through contamination of a skin wound and may affect both immunocompetent and immunocompromised patients [6]. Systemic diseases due to *Exophiala* spp. have been reported [4, 7].

The optimal treatment of these infections is controversial. The surgical excision with administration of topical iodide or systemic amphotericin B, with or without flucytosine, is usually successful [8]. Ketoconazole has been used as an alternative to flucytosine [8, 9] and itraconazole treatment may be effective for some patients with chromoblastomycosis and phaeohyphomycosis [10, 11].

We tested the *in vitro* susceptibility of 11 clinical isolates of *Exophiala* spp. to six antifungal drugs.

Materials and methods

Isolates

A set of 11 clicinal isolates of *Exophiala* spp. (nine *Exophiala spinifera*, one *Exophiala dermatitidis*, one *Exophiala castellanii)* were evaluated in this study. The isolates were frozen in glycerol at −70 °C before they were tested. As quality controls *Candida parapsilosis* (ATCC 22019), *Candida krusei* (ATCC

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6258) and *Paecilomyces variotii* (ATCC 22319) were used.

Drug dilutions

The minimal inhibitory concentrations (MICs) of the antifungals were determined by the microdilution method according to the proposed NCCLS standard [12] in sterile, flat-bottomed, 96-well microtitre plates. The drugs that were used in this test were: miconazole (Janssen Research Foundation, Beerse, Belgium), terbinafine (Novartis, Basel, Switzerland), itraconazole (Janssen Research Foundation), UR 9825 (Ureach, Madrid, Spain), voriconazole (Pfizer, Central Research, Sandwich, UK), and amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands). All drug solutions were freshly prepared. Miconazole and UR 9825 were dissolved at a concentration of 3200 $\mu g\,ml^{-1}$ in dimethyl sulphoxide (DMSO), terbinafine was dissolved first in DMSO with 5% Tween 80 followed by adding the same volume of DMSO so that the final concentration of terbinafine was 1600 $\mu g\,ml^{-1}$, itraconazole was dissolved at a concentration of 3200 $\mu g\,ml^{-1}$ in 0.2 M HCl/acetone (50%/50%), voriconazole was dissolved at a concentration of 3200 $\mu g\,ml^{-1}$ in water with 10% dimethylformamide and amphotericin B was dissolved at a concentration of 5000 $mg\,l^{-1}$ in sterile water. Each drug was serially twofold diluted in RPMI 1640 to obtain two times the final concentration which ranged from 64 to 0.06 $\mu g\,ml^{-1}$ for miconazole, 32 to 0.03 $\mu g\,ml^{-1}$ for terbinafine, itraconazole, UR 9825 and voriconazole and 16 to 0.015 $\mu g\,ml^{-1}$ for amphotericin B. The first concentration of itraconazole was made in water and not in RPMI 1640 in contrast with the other dilutions of drugs. Therefore wells 1–11 contained a series of drug dilutions in 100 µl volumes. Well 12 contained drug-free medium and served as the growth control. The microtitre plates were kept at $-70\,^{\circ}C$.

Medium

The medium used was liquid RPMI 1640 (GIBCO BRL, Life Technologies, The Netherlands) with L-glutamine without sodium bicarbonate and supplemented with 0.165 M MOPS buffer. The pH of the medium was adjusted to 7.0 ± 0.1 at $22\,^{\circ}C$.

Inoculum preparation

Each isolate had been grown for 7 days on Sabouraud glucose agar with 0.5% chloramphenicol at room temperature and was then subcultured on the same medium for 5–7 days at $37\,^{\circ}C$. Spores from these cultures were collected with a cotton stick suspended in sterile water. After the heavy particles were allowed to settle the turbidity of the supernatant was measured spectrophotometrically (Spectronic 20D; Milton Roy, Rochester, NY, USA) at 530 nm and its transmittance was adjusted to 68 to 70%. Each suspension was diluted 1 : 50 in RPMI 1640 to obtain two times the final inoculum size.

Incubation and MIC determination

After adding the inocula the microtitre plates were agitated for 15 s and incubated for 48 and 72 h at $37\,^{\circ}C$. The MIC was determined visually as the lowest concentration showing 75% visible growth inhibition compared with the growth of the drug-free control. For amphotericin B only, the MIC was determined as the lowest concentration showing 100% visible growth inhibition. For isolates with MICs outside scale, the lowest and the highest drug concentration, respectively, were considered.

Data analysis

The ranges and geometric means of MICs as well as the MIC_{90} and MIC_{50} were determined for each drug.

Results

The growth for most of the isolates was adequate at 48 h and for all after 72 h. The MIC range, geometric means, MIC_{90} and MIC_{50} for the isolates of *E. spinifera* after 48 h and 72 h incubation are summarized in Table 1. All the isolates of *E. spinifera* were very susceptible to the six drugs that were used since the $MICs_{90}$ for all drugs were lower than 1 $\mu g\,ml^{-1}$. The isolates were less susceptible for miconazole with MICs after 72 h ranging from 0.125 to 1 $\mu g\,ml^{-1}$ and a geometric mean of 0.46 $\mu g\,ml^{-1}$ and for amphotericin B with MICs ranging from 0.125 to 1 $\mu g\,ml^{-1}$ and a geometric mean of 0.37 $\mu g\,ml^{-1}$. The most active drug was terbinafine with a geometric mean of 0.06 $\mu g\,l^{-1}$ and the MIC_{90} of 0.125 $\mu g\,ml^{-1}$ and voriconazole with a geometric mean of 0.08 $\mu g\,l^{-1}$ and MIC_{90} of 0.25 $\mu g\,ml^{-1}$.

The antifungal activities of the six drugs against the two isolates of *E. dermatitidis and E. castellanii* are shown in Table 2. Both isolates were very susceptible to the six drugs with MICs lower than 0.5 $\mu g\,ml^{-1}$. The most active drug was terbinafine whose MICs were 0.03 $\mu g\,ml^{-1}$ for both isolates.

Table 1. Antifungal activities of miconazole, terbinafine, itraconazole, UR 9825, voriconazole, and amphotericin B against nine clinical isolates of *Exophiala spinifera*

Incubation time	Antifungal agent	MIC range ($\mu g\ ml^{-1}$)	G mean ($\mu g\ ml^{-1}$)	MIC_{50} ($\mu g\ ml^{-1}$)	MIC_{90} ($\mu g\ ml^{-1}$)
After 48 h	Miconazole	0.5–1	0.660	0.5	1
	Terbinafine	0.03–0.06	0.040	0.03	0.06
	Itraconazole	0.125–0.25	0.165	0.125	0.25
	UR 9825	0.03–0.5	0.142	0.125	0.5
	Voriconazole	0.03–0.25	0.070	0.06	0.25
	Amphotericin B	0.06–0.5	0.188	0.25	0.5
After 72 h	Miconazole	0.125–1	0.463	0.5	1
	Terbinafine	0.03–0.125	0.056	0.06	0.125
	Itraconazole	0.03–0.5	0.157	0.125	0.5
	UR 9825	0.03–0.5	0.144	0.125	0.5
	Voriconazole	0.03–0.25	0.077	0.125	0.25
	Amphotericin B	0.125–1	0.367	0.25	1

Table 2. Antifungal activities of miconazole (MCZ), terbinafine (TB), itraconazole (ITZ), UR 9825 (UR), voriconazole (VRZ), and amphotericin B (AB) against *E. dermatitidis* and *E.castellanii*

Species	Incubation time	MCZ ($\mu g\ ml^{-1}$)	TB ($\mu g\ ml^{-1}$)	ICZ ($\mu g\ ml^{-1}$)	UR ($\mu g\ ml^{-1}$)	VCZ ($\mu g\ ml^{-1}$)	AB ($\mu g\ ml^{-1}$)
E. dermatitidis	48 h	0.06	0.03	0.06	0.125	0.06	0.25
	72 h	0.06	0.03	0.06	0.125	0.125	0.5
E. castellanii	48 h	0.5	0.03	0.25	0.06	0.06	0.5
	72 h	0.5	0.03	0.25	0.125	0.06	0.5

Discussion

Mycoses caused by *Exophiala* species are uncommon despite the wide geographic distribution of these fungi. Most patients that presented with phaeohyphomycosis or chromoblastomycosis were treated with surgical excision combined with antimycotic treatment [2, 3]. There is however, no standard therapy and the responses to different treatment regimens were variable. Response to treatment appears to depend on the time of diagnosis. One patient with an early diagnosis responded to surgical excision alone [13] whereas others have died because the diagnosis was not made during life [14].

Several drug regimens have been used for the treatment of infections by *Exophiala* species including amphotericin B in combination with ketoconazole, ketoconazole, itraconazole combined with 5-flucytosine [15], and itraconazole monotherapy. One patient failed to treatment with ketoconazole but responded to oral itraconazole [16]. Some authors consider itraconazole to be the drug of choice for the treatment of patients with phaeohyphomycosis [17]. However, in one patient with chromoblastomycosis caused by *E. spinifera* the lesions progressed during therapy with itraconazole, and this was associated with a rise of the MIC of the strain although the rise was not significant [18]. Alternatively, the absorption of itraconazole capsules may be variable and clinical failure may have been due to insufficient plasma levels.

The present results show that clinical isolates of *Exophiala* are susceptible to all the antifungal drugs that were evaluated. Interestingly, terbinafine appeared to be very active *in vitro* against *Exophiala* species. Clinical experience with terbinafine in the treatment of patients with phaeohyphomycosis or chromoblastomycosis is limited. In an open trial treatment of chromoblastomycosis with terbinafine at a dosage of 500 mg day^{-1} resulted in a spectacular improvement of lesions [18]. Moreover, cure was observed in imidazole-refractory patients or chronic cases [18]. Terbinafine appeared to be inactive in an experimental model in which mice were challenged with three agents of central nervous system phaeohyphomycosis namely *Cladophialophora bantiana*, *Ochroconis constricta* and *Exophiala dermatitidis* [19]. However, terbinafine is metabolized very rapidly in rodents and low drug levels may account for the poor efficacy. Nevertheless, terbinafine may be a promising agent since the drug achieves excellent levels in the skin. Moreover, the drug may be synergistic when combined with azoles. Terbinafine acts within the

same pathway as the azoles and synergistic activity has been reported for terbinafine and itraconazole against *Scedosporium prolificans* [20].

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