

***In vitro* activity of amphotericin B, itraconazole, terbinafine and 5-fluocytosine against *Exophiala spinifera* and evaluation of post-antifungal effects**

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Antifungal susceptibility profiles were determined for 16 strains of the black yeast *Exophiala spinifera* applying different temperature regimens. Fluconazole was the least effective *in vitro*. Lowest minimal inhibitory concentration (MIC) values were found with itraconazole. The activities of antifungal agents against environmental and clinical strains were similar. Post-antifungal effect (PAFE) of four drugs was determined for 11 clinical strains. PAFE was observed only for amphotericin B, with extended inhibition times seen at high drug concentrations.

Keywords antifungals, black yeasts, *Exophiala*, PAFE

Introduction

Black yeasts and their filamentous relatives in the order Chaetothyriales are found in the environment in a diversity of niches such as soil, fresh water, fungal fruiting bodies, moist tiles, surfaces of fruits and creosote-treated wood. Their abundance is usually very limited, however, and they are therefore isolated only when selective techniques are applied. In comparison with this low density in nature, the occurrence of these fungi in clinical settings is remarkably high. Currently 30 species have been reported to be involved in mycoses of humans and other vertebrates [1]. In the past, the slow-growing black colonies of Chaetothyriales were frequently discarded as purported saprobic contaminants, but it is increasingly realized that these fungi may be the primary cause of a wide array of infections. The mycoses reported range from mild cutaneous forms to devastating systemic infections that are mostly fatal.

Exophiala spinifera is known as aetiological agent of disseminated infection [2], causing chronic systemic infections with disfiguring secondary cutaneous lesions

in children and adolescents. Such infections are nearly always fatal [3–5]. However, about half of the strains held in collections were recovered from the environment [6]. Direct transmission from environmental sources to the symptomatic patient has not yet been proved. Two possibilities exist. Clinical strains may be genotypically different from their environmental counterparts, e.g. in virulence, thermotolerance, ability to grow in submersion or susceptibility to antimycotics. Alternatively, environmental strains may have genetic make-up and pathogenic potential similar to those of their clinical counterparts, and their occurrence in a habitat other than a human host may be coincidental. Clinical and environmental strains of the same species then should be treated with similar precautions. One of the aims of the present study is to investigate whether there are differences in antifungal drug susceptibility between strains from the environment and strains from proven cases of human infection.

The correlation between minimal inhibitory concentration (MIC) values obtained according to criteria of the National Committee of Clinical and Laboratory Standards [7] and the clinical response of infected patients remains problematic. MIC values, determined in a system in which the fungus is exposed to a constant drug concentration, may be misleading with regard to the *in vivo* situation, where organisms usually experience fluctuating drug levels [8]. Using an animal model for *Aspergillus* infection, it was found that this *in vivo*

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fluctuation is especially important in amphotericin B therapy, but less important for itraconazole and voriconazole treatment [8].

The study of post-antifungal effects (PAFE) could be helpful in determining the overall effect of antifungal drugs, as such studies simulate the *in vivo* situation in which fluctuating drug levels occur. In the present study, antifungal drug susceptibilities were studied comparing environmental and clinical strains. PAFE was also evaluated, being the first study of this effect in a member of the 'black yeasts' (anamorphic *Herpochytriaceae*), exemplified by *E. spinifera* strains.

Materials and methods

Strains and chemicals

Eight clinical and eight environmental well-documented isolates of *E. spinifera* were used (Table 1). For PAFE 3, further clinical isolates were added. Drugs tested were amphotericin B (AMB) (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (ITZ) (Janssen-Cilag, Beerse, Belgium), voriconazole (VCZ) (Pfizer Central Research, Sandwich, UK), terbinafine (TBF) (Novartis, Basel, Switzerland), fluconazole (FCZ) (Pfizer Central Research, The Netherlands) and 5-fluorocytosine (5-FC) (ICN Pharma BV, Zoetermeer, The Netherlands). Aliquots of stock solutions were stored at $-70\text{ }^{\circ}\text{C}$ until used. All drugs with

the exception of FCZ were dissolved in dimethylsulphoxide (DMSO) at concentrations of $3200\text{ }\mu\text{g ml}^{-1}$ for AMB, ITZ and TBF, $12800\text{ }\mu\text{g ml}^{-1}$ for 5-FC, and $1600\text{ }\mu\text{g ml}^{-1}$ for VCZ. FCZ was dissolved in water at concentration of $25600\text{ }\mu\text{g ml}^{-1}$. Twofold serial dilutions of the drugs were made in RPMI-1640 medium (GIFCO BRL, Woerden, The Netherlands) in order to obtain final concentrations that ranged from 0.015 to $16\text{ }\mu\text{g ml}^{-1}$ for AMB, ITZ and TBF; 0.063 to $64\text{ }\mu\text{g ml}^{-1}$ for 5-FC and 0.125 to $128\text{ }\mu\text{g ml}^{-1}$ for FCZ. RPMI 1640 medium (with l-glutamine, without bicarbonate) was buffered to pH 7.0 with 0.165 m morpholinopropanesulphonic acid (Sigma-Aldrich, Steinheim, Germany).

Susceptibility testing

Isolates were subcultured serially at intervals of 5–7 days at $28\text{ }^{\circ}\text{C}$ on potato glucose agar (PGA) to obtain adequate sporulation. Conidia were collected with a cotton swab and suspended in saline with 0.5% Tween 20. After the heavy particles were allowed to settle, concentrations of conidia were determined microscopically by haemocytometer and adjusted to obtain a suspension of 10^6 conidia ml^{-1} . Viability was confirmed by plating serial dilutions on Sabouraud glucose agar (SGA) incubated at $28\text{ }^{\circ}\text{C}$ and determining colony counts. Inocula were prepared from conidial suspensions diluted 1:100 to a final concentration of 0.5×10^4 to 4.5×10^4 cfu ml^{-1} .

A broth microdilution method was applied according to the NCCLS guideline M38-P [7]. Tests were performed in 96-well flat-bottom microtitration plates (Corning, Ames, MA, USA) which were kept at $-70\text{ }^{\circ}\text{C}$ until the day of testing. Conidial suspensions were diluted in RPMI 1640 to obtain two times the desired inoculum concentration. A drug-free well containing 0.01% DMSO in the medium served as the growth control. Plates were incubated at 28, 35 and $37\text{ }^{\circ}\text{C}$ for 48, 72 and 96 h. The MICs were determined using a spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex, Salzburg, Austria). The relative optical densities (ODs) for each well, based on measurements at 405 nm, were calculated (%) based on the following equation: $[(\text{OD of drug-containing well} - \text{background OD}) / (\text{OD of drug-free well} - \text{background OD})] \times 100\%$. The MIC for amphotericin B was defined as the lowest concentration of the drug that showed at least 95% reduction of growth compared with that of the growth control (MIC-0). For the remaining drugs, the MIC was defined as the lowest concentration of the drug that showed 50% reduction of growth compared

Table 1 Source and numbers of the strains tested

CBS	Other reference	Source
425.92		Heated apple juice
	dH 11324	Maize
236.93		Apple juice
669.76		Palm fruit
	IFM 41692	Bark
	IFM 41856	Soil
	IFM 41846	Cactus
667.76		Fallen palm
194.61		Disseminated
899.68	ATCC 18218	Nasal granuloma
	dH 11328	Skin
	dH 11326	Skin
356.83		Skin
	dH 12309	Chromoblastomycosis
	dH 11327	Head lesion
269.28		Skin

For detailed isolation data, see De Hoog *et al.* [6] and Vitale & De Hoog [13].

ATCC, American Type Culture Collection, Manassas, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH, G. S. de Hoog private collection; IFM, Research Institute for Pathogenic Fungi, Chiba, Japan.

with that of the growth control (MIC-2). All assays were done in duplicate.

PAFE assay

For PAFE 11 clinical isolates of *E. spinifera* were tested with AMB, ITZ, TBF and 5-FC applying the method adapted for use in filamentous fungi [9]. AMB, ITZ, TBF were dissolved in DMSO at initial concentrations of $400 \mu\text{g ml}^{-1}$ and 5-FC in water at an initial concentration of $3200 \mu\text{g ml}^{-1}$. Stock solutions were stored at -70°C until used. Then they were diluted in buffered RPMI 1640 medium. Serial dilutions of the drugs were made in buffered RPMI 1640 at final concentrations of $4\times$ and $1\times$ the corresponding MIC for each strain for AMB and 5-FC, and $10\times$ the MIC for ITZ and TBF. Control conidial suspensions were made in buffered RPMI 1640. Inocula were prepared as described above. The concentration of conidia was established microscopically by haemocytometer Bürker-Türk chambers. Final concentration was adjusted to 4×10^5 cfu ml^{-1} . Samples were incubated for 4 h with continuous shaking at 37°C , washed three times with saline and centrifuged at $3500\times g$ for 15 min. Ninety-eight per cent of the supernatant was decanted and the sediment was resuspended in 10 ml of buffered RPMI-1640. A procedure with two washings and removal of 90% of supernatant has been shown to reduce the antimicrobial concentration 100-fold, whereas with two washings and complete decanting the concentrations are reduced 10 000-fold [10]. Following this step, 100 μl of sample was diluted 10-fold in sterile water and 30- μl aliquots were plated on SGA for colony count determination, and incubated at 37°C . The concentration of viable cfu ml^{-1} for exposed conidia was determined in order to verify the viability after drug exposure and to compare the cfu ml^{-1} both control with exposed cells; 200 μl of the resuspended suspensions were transferred to microtitration plates which were incubated at 37°C in a computerized spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex Instruments GmbH, Salzburg, Austria). Growth measured as change in turbidity at 405 nm was monitored automatically at 10-min intervals for 96 h. All assays were performed in duplicate.

Data analysis

Effects of drugs at different temperatures and incubation periods were analysed for each isolate group (environmental and clinical) by ANOVA (Friedman test) and comparison between these by *T*-test (Mann-

Whitney test). *P* values of <0.05 were considered to be statistically significant.

PAFE was calculated using the point in the growth curve (OD_0) that corresponds with the first significant increase in OD as was described previously [9] by using the formula $\text{PAFE} = T - C$, where *T* was the time of the first significant increase in OD_0 of the exposed spores after removal of the drug and *C* was the time of the first significant increase in OD_0 of the control. Thus, PAFE was defined as the difference in time (Δt) between exposed and controls to reach the defined point in the growth curve and was expressed in hours. The time to reach this chosen point OD_0 of at least six controls for each species was calculated and the mean, range, upper 95% confidence interval (CI) and the coefficient of variation were calculated in order to determine the reproducibility of the control curves at that point and to establish the reproducibility of the experiments. For each species, the upper 95% CI of the controls was chosen as the cut-off level that distinguished between presence or absence of PAFE.

When re-growth of the exposed isolates occurred within the upper 95% CI time-frame of the controls, PAFE was considered to be absent. Alternatively, if re-growth was delayed following drug exposure and the lower 95% CI of the exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered to be present. Growth curves of each exposed isolate were compared only with pooled controls from that same isolate.

Results

The geometric means and the range of MICs obtained after 72 h are given in Table 2. No significant differences were found in MICs after 72 and 96 h of incubation (data not shown). The most active drug was ITZ, against both environmental and clinical strains, regardless of the temperature of incubation with geometric mean 0.04 and $0.06 \mu\text{g ml}^{-1}$, respectively. VCZ (geometric means 0.4 and $0.2 \mu\text{g ml}^{-1}$, respectively) and TBF (geometric mean 0.2 and $0.3 \mu\text{g ml}^{-1}$, respectively) at 35°C were also inhibitory. AMB susceptibility values varied among the isolate classes, environmental and clinical, with geometric mean at 35°C of 0.3 for environmental isolates and 0.9 for clinical isolates. Differences were also seen in 5-FC values, which were 1.83 for environmental and 5.66 for clinical isolates. FCZ showed little effect but some strains showed MIC values of 8–16 and $32 \mu\text{g ml}^{-1}$ at all temperatures.

Statistical analysis was performed for each drug under different incubation regimens. Susceptibilities

Table 2 MICs of environmental and clinical strains at different temperatures after 72 h

Drugs	Environmental						Clinical					
	28 °C		35 °C		37 °C		28 °C		35 °C		37 °C	
	Gm	Range	Gm	Range	Gm	Range	Gm	Range	Gm	Range	Gm	Range
AMB	0.77	0.125-4	0.35	0.125-0.5	0.59	0.125-2	1.30	0.25-4	0.92	0.5-2	1.09	0.5-4
FCZ	26.91	16-64	41.50	16-128	38.05	16-64	32.00	8-64	45.25	16-128	38.05	8->128
VCZ	0.25	0.125-0.5	0.46	0.125-4	0.25	0.063-0.5	0.28	0.031-1	0.27	0.031-0.5	0.25	0.063-0.5
TBF	0.12	0.031-0.5	0.25	0.031-2	0.30	0.031-1	0.63	0.5-1	0.30	0.031-1	0.35	0.063-1
5-FC	3.17	1-8	1.83	0.125-64	1.83	0.25-16	2.69	0.25-16	5.66	0.5-64	5.19	1-16
ITZ	0.02	0.016-0.031	0.04	0.016-0.125	0.05	0.016-0.125	0.07	0.031-0.125	0.06	0.031-0.125	0.05	0.016-0.125

Gm, geometric mean.

of environmental strains tested (i) after incubation periods of 72 and 96 h were compared at 28, 35 and 37 °C. The same was done (ii) among the clinical strains, and finally pooled data of environmental and clinical strains (iii) were compared: for (i) and (ii) no statistically significant differences were observed ($P > 0.05$); for (iii) no statistically significant differences were found between incubation periods, but a temperature-related difference was observed with AMB ($P < 0.05$) between environmental and clinical strains.

Eleven clinical isolates were selected for PAFE experiments. The MIC ranges were from 0.5 to 2 $\mu\text{g ml}^{-1}$ for AMB, from 0.031 to 0.125 $\mu\text{g ml}^{-1}$ for ITZ, from 0.063 to 0.5 $\mu\text{g ml}^{-1}$ for TBF and from 0.5 to 64 $\mu\text{g ml}^{-1}$ for 5-FC. When PAFE was present, the growth curve of the exposed conidia shifted to the right compared with that of the control (Fig. 1). After 4 h of incubation at 4 \times MIC, all strains displayed significant PAFE with AMB with a mean of 16.2 h and a range of 9.7-19 h. An example of PAFE observed in strain dH 12309 is shown in Fig. 1. For 1 \times MIC, 7/11 strains tested showed PAFE, with a mean of 9.8 h and a range of 1.6-16 h (Table 3). For the other drugs, PAFE was not observed (data not shown). The growth curve after exposure to ITZ for strain dH 12309 is given in Fig. 2, where PAFE was not observed at 4 \times and 10 \times MIC.

Discussion

Our antimycotic susceptibility data for AMB and VCZ are similar to those given by McGinnis & Pasarell [11], but we obtained higher susceptibility values for ITZ. Our ITZ, TBF, VCZ and AMB MIC values matched those of Meletiadis *et al.* [12].

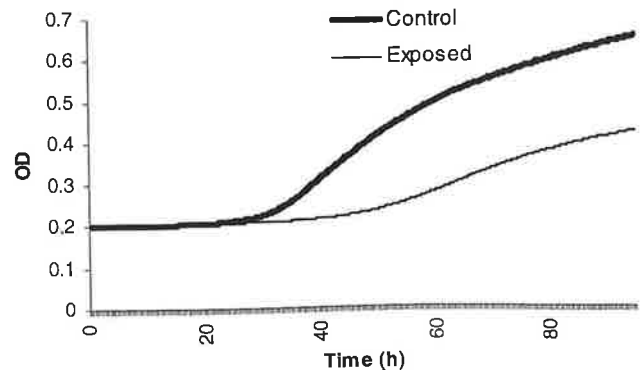


Fig. 1 PAFE of 18.4 h observed in strain dH 12309 after exposure to amphotericin B for 4 h at 4 \times MIC.

Table 3 PAFEs induced by exposure of *E. spinifera* to amphotericin B at concentrations of 4 × and 1 × MIC, after 4 h of exposure using OD₀ criteria

Strain	Control (U 95% CI)	4 × MIC (L 95% CI)	PAFE (h)	1 × MIC (L 95% CI)	PAFE (h)
12309	35.6	47.9	18.4*	36.1	7.7*
194.61	40.6	43.4	19.0*	43.9	16.0*
12308	55.7	62.0	10.7*	54.5	6.2
12301	56.7	57.4	9.7*	54.1	8.9
356.83	39.0	47.4	19.0*	42.8	13.5*
12304	46.3	57.9	19.0*	49.6	14.5*
11328	42.3	49.9	18.6*	49.0	13.1*
899.68	57.7	63.9	16.4*	59.3	10.6*
11327	12.2	25.3	16.5*	10.5	10.2
12312	18.1	34.7	13.0*	18.7	6.0*
269.28	24.8	39.3	18.0*	13.3	1.6

U 95% CI: upper 95% confidence interval; L 95% CI: lower 95% confidence interval.

*Statistically significant PAFE.

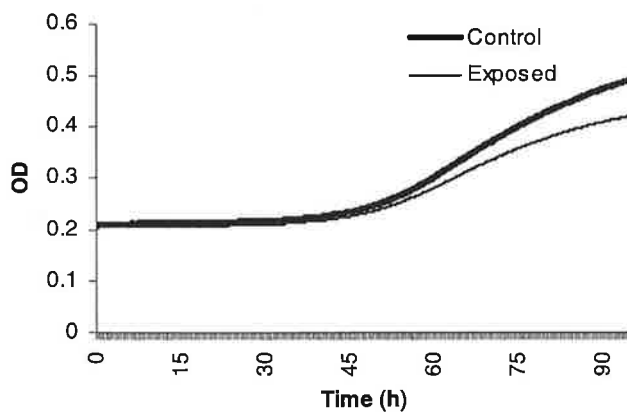


Fig. 2 PAFE absent from strain dH 12309 after exposure to itraconazole for 4 h at 4 times MIC. The same graph was obtained after exposure to drug at 10 × MIC.

Because *E. spinifera* is encountered in soil and in association with plant material such as maize ears and fruit juices, it is presumably a saprotrophic fungus. However, about half of the strains collected to date were obtained as agents of skin and soft-tissue infections or of fatal, systemic infections [6]. Traumatic cutaneous or subcutaneous infections have mainly been observed in adults, whereas systemic cases, for which no portal of entry has been defined, have occurred only in children and adolescents. This remarkable ecological and clinical spectrum of *E. spinifera* raises the possibility that some strains may be more tolerant than others of higher temperatures. Our results, however, show that susceptibilities to antifungal agents generally were not influenced by temperature. The only exception was a small but statistically significant difference in activity of AMB at different temperatures (Table 2). This deviation was based on a single difference of two dilution steps.

Recently, we described a new species, *E. attenuata*, which is morphologically similar to *E. spinifera* and which as yet has not been reported to cause infection in humans [13]. Whether it has an opportunistic potential to humans similar to that of *E. spinifera* is not known. The close phylogenetic kinship of the two fungi suggests this potential. The aetiological agent of a case in a cat ascribed to *E. spinifera* by Chermette *et al.* [14] was later recognized to be *E. attenuata* by Vitale & De Hoog [13]. The significance of phylogeny for predicting the clinical significance of fungal species was stressed earlier by De Hoog [15] and McGinnis & Pasarell [11]. De Hoog [16] used phylogenetic relationships with known human pathogens as one of the criteria to assign fungal species to a particular BioSafety Level. In view of this predicted opportunistic potential, we included environmental *E. spinifera* isolates in our study.

Therapeutic measures used in cases of *E. spinifera* infection have been highly diverse, including surgical excision, systemic use of antifungals or a combination of both. Relapses have frequently been observed, and therefore long-term antimycotic therapy seems mandatory. The optimal treatment is still in dispute [17] and the efficacy of applied antifungal therapy regimens is variable. Rajam *et al.* [3], Lacaz *et al.* [18], Dai *et al.* [4] Dieye *et al.* [19], Wang *et al.* [20], Mirza *et al.* [21], Campos *et al.* [22], Padhye *et al.* [5] and Lacroix *et al.* [23] refrained from use of any therapy, whereas Nielsen & Conant [2], and Gold *et al.* [24] used surgery alone. A chromoblastomycosis-like infection reported by Barba *et al.* [25] was successfully treated with 200 mg ITZ daily for 12 months. The isolate involved had an *in vitro* MIC value of 0.031 µg ml⁻¹. Padhye *et al.* [26] in treating a similar case caused by strain CDC B-5383 used cryosurgery followed by KTZ (up to 400 mg day⁻¹), 5-FC and FCZ, all without success. The *in*

vitro data showed that the strain had an MIC of $64 \mu\text{g ml}^{-1}$ for FCZ, $2 \mu\text{g ml}^{-1}$ for 5-FC and $0.125 \mu\text{g ml}^{-1}$ for ITZ. Clinical improvement was obtained with the combination of ITZ and 5-FC, but the patient later relapsed. A subcutaneous lesion caused by isolate CDC B-3868 was treated by Padhye *et al.* [27] using KTZ monotherapy. The dose was raised from 200 to 400 mg day⁻¹ during treatment. After 6 weeks, growth of the isolate was again obtained from the lesion, and hence the therapy was changed to KTZ (600 mg day^{-1}) plus 5-FC (2 g day^{-1}). The lesion resolved after 1 month. Our present data showed a 5-FC MIC value of $4 \mu\text{g ml}^{-1}$ for the same strain; hence the healing that occurred should be ascribed to KTZ. Sharkey *et al.* [28] described cases of phaeohyphomycoses by members of various dematiaceous genera where treatment with amphotericin B, ketoconazole or miconazole appeared inadequate. Among these cases was an infection in a joint due to *E. spinifera*. A total dose of 450 mg of amphotericin B was given within a 1 month which led to improvement, although subsequent cultures remained positive after azole therapy. Ketoconazole was given at 100 mg day^{-1} for 8 months, and subsequently 400 mg day^{-1} for 4 further months, but progression was observed. Further, itraconazole with a daily dose of 100 mg was administered, but total remission after 8 months was observed. The *in vitro* MIC for this strain was $0.15 \mu\text{g ml}^{-1}$. Kotylo *et al.* [29] administered KTZ 200 mg twice daily to their patient, but the lesion did not improve. They then performed surgery followed by ITZ therapy of 50 mg day^{-1} for 6 weeks, then 100 mg day^{-1} for 5 weeks. This treatment was successful. Negroni *et al.* [30] documented a patient with a skin lesion and adenopathy who was treated with ITZ 5 g day^{-1} and 5-FC 200 mg day^{-1} for 8 months. Improvement was noted, but 2 years later, relapse occurred. The same was observed with ITZ 200 mg day^{-1} and 5-FC 6 g day^{-1} for 4 months, even after the ITZ dosage was raised to 400 mg day^{-1} . AMB and liposomal AMB were then started but discontinued due to serious adverse effects. Thereafter, 400 mg day^{-1} ITZ, 6 g day^{-1} 5-FC and 300 mg day^{-1} ranitidine were given for 8 months, leading to remission. The *in vitro* MIC values for this strain were $0.031 \mu\text{g ml}^{-1}$ for ITZ and $4 \mu\text{g ml}^{-1}$ for 5-FC. Treatment of two cases in cats [14,31] was unsuccessful. It should be noted, that the causative agent in the case reported by Chermette *et al.* [14] has been re-identified as *E. attenuata* Vitale *et al.* [13]; the strain of Kettlewell *et al.* [31] is not known to be preserved.

In vivo antimycotic data for *E. spinifera* are scant. Most existing studies are based on older, less effective compounds. The overview given above clearly illus-

trates the persistent nature of infections caused by *E. spinifera*. Relapse is common despite prolonged treatment, and poor resolution is frequently obtained despite relatively promising MIC values for the drug used. Correspondence of *in vitro* data to *in vivo* efficacy seems to be low.

Post-antibiotic effect (PAE), i.e. persisting suppression of growth of a microbe after limited exposure to an antibiotic compound, may simulate the situation *in vivo* better than standard MIC testing does, as the organism is exposed only temporarily to an inhibitory drug level. The PAE effect was first reported with bacteria [10]. It was found to be dependent on several factors such as concentration of the drug, exposure time, inoculum and type of medium. Later, similar phenomena were observed in fungi and referred to as post-antifungal effect (PAFE) [9,32]. In our study, we found that PAFE was dependent on concentration. This is slightly different from data reported in *Aspergillus* [9], with *Exophiala* longer values of PAFE being obtained. *Candida* species also displayed relatively long PAFEs when exposed to amphotericin B at concentrations above the MIC, and relatively short PAFEs after exposure to sub-MIC drug levels [32]. The inability of itraconazole to induce PAFE in *Exophiala* is in accordance with observations reported for *Aspergillus* and *Candida* where no measurable PAFE was observed following exposure to azoles [9,32,33]. The absence of PAFE mediated by these drugs may be related to the different mechanisms by which the drugs act. AMB acts directly by binding to sterols in the fungal cell and altering membrane permeability, which leads to cell death, whereas azoles or allylamines interfere with the biosynthesis pathway of ergosterol. The exposure times applied in PAFE testing may be too short to allow these drugs to exert a significant action. PAFE was not observed when *E. spinifera* was tested with 5-FC. PAFE was induced with 5-FC PAFE in *Candida* [34]. The discrepancy is perhaps explained by the fact that these authors used YNB media rather than RPMI-1640. YNB was found as more nutritious media provided highest growth compared with RPMI-1640 [35]. The action of the drug is dependent in the fungi growth, so might be possible that the media influence in the presence of PAFE.

PAFE is an alternative *in vitro* susceptibility assay that may provide additional information on the interaction of antifungal agent and fungus. *In vivo* experiments are needed to evaluate the importance of this phenomenon as a method to determine antifungal therapeutic regimens.

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