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Susceptibility profile of clinical isolates of non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species and literature review

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The *in vitro* susceptibility profile of 24 clinical isolates of non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species was analysed. In addition, the susceptibility results of 98 other strains from seven different reports were reviewed. The latter included studies which used antifungal susceptibility testing reference procedures or commercial methods which exhibited high correlation rates with the reference procedures. A total of 122 isolates were analysed (57 *Cryptococcus albidus*, 39 *Cryptococcus laurentii*, ten *Cryptococcus uniguttulatus*, ten *Cryptococcus humicola*, four *Cryptococcus curvatus*, and two *Cryptococcus luteolus*). Amphotericin B was *in vitro* the most active compound against all species, while flucytosine and candins were inactive. Fluconazole exhibited a limited *in vitro* activity, particularly against *C. albidus*, *C. uniguttulatus* and *C. laurentii*. Voriconazole, itraconazole and posaconazole were active against most of isolates, but we found significant rates of decreased susceptibility. Identification and susceptibility testing of *Cryptococcus* spp. should be performed on a routine basis in view of their unpredictable susceptibility profiles.

Keywords Basidiomycetous, echinocandins, amphotericin B, emerging pathogens

Introduction

The genus *Cryptococcus* comprises several species which are able to cause infections in human and animals, e.g., superficial and central nervous system mycoses, as well as pulmonary diseases. *Cryptococcus neoformans and Cryptococcus gattii* are the major pathogens within the genus. Other *Cryptococcus* species have classically been considered to be non-pathogenic. However, *Cryptococcus albidus*, *Cryptococcus laurentii*, *Cryptococcus luteolus*, *Cryptococcus uniguttulatus*, *Cryptococcus curvatus* (former *Candida curvata*), and *Cryptococcus humicola* (former *Candida humicola* and *Cryptococcus humicolus*), have emerged as opportunistic pathogens over the last few years [1]. The increase of infections caused by these emerging species may be related to several factors, especially to the rise of immunosuppressed patients who are more susceptible to opportunistic fungal infections. In addition, although the wide use of antifungals has efficiently reduced the incidence of the most prevalent pathogenic fungi, it has also favoured the appearance of niches for rare and may be more resistant species.

Pigeons and other birds are the most important reservoirs for *Cryptococcus* species [2,3] and infections caused by them are frequently related to the exposure to avian droppings. *C. albidus* is probably the second most common pathogen wihin this genus and its inhalation seems to be its main route of entry. *C. albidus* has been isolated from eye and cutaneous lesions [4,5], and from disseminated infections in patients with lymphoma or with leukaemia [4–9].

Human cases of *C. laurentii* infections have also been reported, including disseminated disease with invasion of

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the central nervous system [10], fungemias in neonates and cancer patients [11,12], oropharyngeal infections in leukaemia patients [13] and a case of pneumonia and pleural effusion in a patient with AIDS [14]. Other species have been isolated from environmental sources but seldom from patient specimens. *C. uniguttulatus* has been involved in onychomycosis and occasionally in systemic infections [15,16]. Infections of the central nervous system in HIV patients caused by *C. humicola* have been described in the literature [17]. This organism has also been involved in systemic infections in patients suffering from cancer and other predisposing diseases [18–20]. Finally, one case of myeloradiculitis in an AIDS patient caused by *C. curvatus* was reported years ago [21].

To gain insights into the management of these emerging infections, we have analysed the antifungal susceptibility profile of 24 clinical isolates of non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species. In addition, we present a review of the literature on the *in vitro* susceptibility results of these species.

Material and methods

A collection of 24 isolates including *C. laurentii*, *C. albidus*, *C. curvatus* and *C. humicola* were used in the study. All strains were recovered from 1996 to 2006 from different Spanish hospitals. The number and origin (sorted by species) of the isolates were as follows: (i) Ten strains of *C. albidus* – two from blood cultures, one from a biopsy, five from skin, one from urine and one from a respiratory sample; (ii) Eight strains of *C. laurentii* – four from skin, one from cerebrospinal fluid, two from blood cultures and one from a deep biopsy; (iii) Two *C. curvatus* strains – both from vaginal exudates; and (iv) four strains of *C. humicola* – three from skin samples and one from a blood culture. *C. uniguttulatus* and *C. luteolus* isolates were not found among our collection.

Each isolate, obtained from a different patient, was sent to the Mycology Reference Laboratory of the National Centre for Microbiology of Spain for identification and susceptibility testing. Isolates were identified by biochemical and morphological characterization [22] and were labelled as CNM-CL (Spanish National Center Microbiology-yeast culture collection) followed by an identification number.

The susceptibility testing was performed following the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing [23]. In order to improve the growth of some organisms, we made minor modifications in the procedure. Briefly, the microplates were wrapped with film sealer to prevent medium evaporation, attached to an electrical driven wheel inside the incubator, shaken at 350 rpm and incubated at 30°C for 48 h. This modification has been shown to improve the growth of non-fermentative yeasts in the microdilution plates [24]. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains.

The antifungal agents used in the study were amphotericin B (ranged 16.0–0.03 mg/l, Sigma Aldrich Quimica S.A., Madrid, Spain), flucytosine (ranged 64.0–0.12 mg/l Sigma-Aldrich), fluconazole (Pfizer S.A., Madrid, Spain), itraconazole (8.0–0.015 mg/l, Janssen S.A., Madrid, Spain), voriconazole (8.0–0.015 mg/l, Pfizer S.A., Madrid, Spain), ravuconazole (8.0–0.015 mg/l, Bristol-Myers Squibb, Princeton, USA), posaconazole (8.0–0.015 mg/l, Schering-Plough, Kenilworth, NJ, USA), terbinafine (16.0–0.03 mg/l, Novartis, Basel, Switzerland), caspofungin (16.0–0.03 mg/l, Merck & Co., Inc., Rahway NJ, USA), micafungin (16.0–0.03 mg/L, Astellas Pharma Inc, Tokyo, Japan), and anidulafungin (16.0–0.03 mg/L, Pfizer S.A.). For amphotericin B, the MIC end points were defined as the lowest drug concentration exhibiting 90% or more reduction in growth as compared with that of the control. The MIC_{90} was calculated when the number of isolates included per species was equal to/or higher than ten. For flucytosine, azole and candin drugs, the MIC end point was defined as 50% of growth inhibition.

In addition, we undertook a MEDLINE search using the keywords '*Cryptococcus* species', '*C. albidus*', '*C. laurentii*', '*C. curvatus*', '*C. humicola*', '*C. uniguttulatus*', '*C. luteolus*', 'antifungal susceptibility testing' and 'emerging yeasts pathogens', as well as text word searching. We included reports available on MEDLINE from 1992, the date of publication of reference procedures for susceptibility testing of yeasts. The review therefore included studies on susceptibility testing performed using the reference methods of CLSI and EUCAST. We also reviewed studies performed with commercial methods such as E-test, Sensititre, YeastOne and ATB fungus, which have exhibited high correlation rates with reference procedures in several comparative investigations.

Results

The MIC values for the quality control strains were in the expected ranges (Table 1). The geometric mean (GM) of MIC values, ranges, and MICs including MIC_{90} values of all antifungal agents tested are shown in Table 2.

All species were susceptible *in vitro* to amphotericin B with GM of MIC values of 0.51 mg/l, 0.17 mg/l, 0.25 mg/l and 0.5 mg/l for *C. albidus, C. laurentii, C. curvatus* and *C. humicola*, respectively. Only one *C. albidus* strain exhibited an amphotericin B MIC value of 2 mg/l. In contrast, flucytosine showed limited *in vitro* activity against the four species, and candins were inactive against all strains.

Table 1 MIC ranges for the control strains. Data are expressed in a range after 30 repetitions and in mg/l

Antifungal agent	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019
Amphotericin B	0.12–0.50	0.12–0.50
Flucytosine	2.0–8.0	0.12–0.50
Fluconazole	16.0–64.0	0.50–2.0
Itraconazole	0.03–0.12	0.03–0.12
Voriconazole	0.06–0.25	0.015–0.06
Posaconazole	0.015–0.06	0.015–0.03
Ravuconazole	0.03–0.12	0.015–0.03
Caspofungin	0.12–0.50	0.50–2.0
Micafungin	0.03–0.12	0.25–1.0
Anidulafungin	0.03–0.12	0.25–1.0

Decreased susceptibility to azole drugs was observed for some isolates. The percentage of strains with decreased *in vitro* antifungal susceptibility included a total of eight strains (80%, 8/10) of *C. albidus* which were found to have an MIC to fluconazole ≥16mg/l, 40% with an itraconazole MIC of ≥1mg/l, 30% presenting a voriconazole MIC of ≥4mg/l, and 30% having a posaconazole MIC value ≥2 mg/l. Half of the *C. laurentii* strains were found to have a fluconazole MIC of ≥16mg/l. In addition, one isolate of this species (12.5%) had a itraconazole MIC of ≥1mg/l and the same percentage showed a voriconazole MIC of ≥4mg/l. One *C. curvatus* strain exhibited a MIC ≥1mg/l to itraconazole but none had a fluconazole MIC ≥16mg/l nor a voriconazole MIC ≥4mg/l. All the strains were susceptible *in vitro* to posaconazole. The percentage of *C. humicola* strains with a fluconazole MIC ≥16mg/l was 50%. Decreased susceptibility was not observed for any of the other azole compounds.

In addition, we have performed a literature review of the reported MIC values of strains of these species. Following the criteria described in Material and Methods, we found seven reports, which described antifungal susceptibility profiles for non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species (Table 3). Most reports included susceptibility profiles for *C. albidus and C. laurentii* clinical and environmental isolates [16,25–28]. Three articles described susceptibility results for two other species, including a total of six *C. humicola* and two

Table 2 Susceptibility results for 24 non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* clinical strains. Data are expressed in mg/l

Antifungal agent		*C. albidus* (n = 10)	*C. laurentii* (n = 8)	*C. curvatus* (n = 2)	*C. humicola* (n = 4)
Amphotericin B	MIC_{90}a	1.0	ND	ND	ND
	Range	0.06–2.0	0.03–0.50	0.25	0.03–1.0
	GM	0.51	0.17	0.25	0.50
Flucytosine	MIC_{90}	>64.0	ND	ND	ND
	range	0.25–>64.0	1.0–>64.0	1.0–64.0	8.0–64.0
	GM	71.1	т64.0	32.5	26.0
Fluconazole	MIC_{90}	>64.0	ND	ND	ND
	range	4.0–>64.0	4.0–>64.0	4.0–8.0	2.0–16.0
	GM	41.2	24.5	6.0	10.5
Itraconazole	MIC_{90}	>8.0	ND	ND	ND
	range	0.12–>8.0	0.06–8.0	0.25–1.0	0.06–1.0
	GM	3.15	1.22	0.62	0.45
Voriconazole	MIC_{90}	8.0	ND	ND	ND
	range	0.12–>8.0	0.06–8.0	0.06–0.25	0.01–0.25
	GM	3.1	1.5	0.15	0.16
Posaconazole	MIC_{90}	>8.0	ND	ND	ND
	range	0.25–>8.0	0.06–0.25	0.06	0.03–0.50
	GM	2.2	0.09	0.06	0.12
Ravuconazole	MIC_{90}	>8.0	ND	ND	ND
	range	0.03–>8.0	0.06–>8.0	0.01	0.03–0.25
	GM	0.50	0.45	0.01	0.10
Caspofungin	MIC_{90}	>16.0	ND	ND	ND
	range	>16.0	>16.0	>16.0	>16.0
	GM	>16.0	>16.0	>16.0	>16.0
Micafungin	MIC_{90}	>16.0	ND	ND	ND
	range	>16.0	>16.0	>16.0	>16.0
	GM	>16.0	>16.0	>16.0	>16.0
Anidulafungin	MIC_{90}	>16.0	ND	ND	ND
	range	>16.0	>16.0	>16.0	>16.0
	GM	>16.0	>16.0	>16.0	>16.0

ND (not determined): MIC_{90} was not calculated if number of isolates was <10. MIC_{90}: MIC including 90% of isolates. GM: Geometric Mean of MIC values

C. curvatus isolates [25,29,30]. Two papers included susceptibility data for *C. uniguttulatus* and *C. luteolus* [16,25].

Our results are in agreement with the published data, regardless of the technique used for susceptibility testing, i.e., CLSI, EUCAST, E-test, ATB fungus or Sensititre-YeastOne. Amphotericin B was found to be the most potent drug against non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species in all reports. Flucytosine had a very limited *in vitro* activity against these species. One report [28] tested micafungin against ten *C. albidus* and ten *C. laurentii* strains and noted that this echinocandin is inactive *in vitro* against the two species.

Regarding azole agents, fluconazole seems to be the least active antifungal against non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species. Itraconazole and voriconazole were active *in vitro* against these organisms. However, significant percentages of decreased susceptibility were observed for *C. albidus* and *C. uniguttulatus* isolates and against some *C. laurentii* strains. No differences in MIC values between clinical and environmental strains were detected apart from the work by García-Martos *et al*., which described that environmental strains were less susceptible than clinical isolates [25].

Discussion

Identification and *in vitro* antifungal susceptibility testing of emerging pathogens has become of increased importance for clinical laboratories as these species often exhibit different susceptibility profiles than more common clinically important fungi. In addition, new therapeutic alternatives with distinct spectra of activities have become available.

Non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species have emerged as human pathogens over the last few years. They have been described as opportunistic pathogens in HIV^+ individuals, as well as in patients with other predisposing factors. *C. albidus* and *C. laurentii* are the most common non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species, with *C. luteolus*, *C. uniguttulatus*, *C. humicola* and *C. curvatus*, less frequently isolated in the clinical setting.

Our study confirms that *C. albidus* and *C. laurentii* are the two non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species with the highest clinical relevance. *Cryptococcus luteolus* and *C. uniguttulatus* isolates were not in our collection. Four *C. humicola* isolates were analyzed, one of them having been isolated in culture from a blood specimen in the course of a systemic infection. In addition, two *C. curvatus* strains were analyzed, both of which were recovered from vaginal exudates of patients with vaginitis.

Regarding the susceptibility profiles, we have used the EUCAST method with minor modifications to obtain the MIC values. It is noteworthy that originally this method was standardized for fermentative yeasts. In the case of non-fermentative yeasts, such as *Cryptococcus* spp., EUCAST is developing a separate method since the growth of these yeasts is impaired by conditions used in the fermentative yeast test. For this reason, a few modifications have been included to improve the measurement of the MIC values. Although there are other methods available to measure antifungal susceptibility profiles, none of them have been adapted for non-fermentative yeasts. For this reason, MIC values obtained cannot be directly compared to MIC values obtained with other methods. Our results and data from the literature indicate that amphotericin B seems to be the therapy of choice to treat *Cryptococcus* infections. *C. albidus* and *C. uniguttulatus* exhibited a decreased susceptibility to fluconazole and a significant number of the strains of both species had high MIC values of itraconazole, voriconazole and posaconazole. *C. laurentii* isolates also exhibited a decreased susceptibility to fluconazole, but most of the isolates were susceptible *in vitro* to other azole agents. The other two species included in the study were more susceptible to azole compounds but the low number of strains analyzed does not permit us to draw any firm conclusion as to the susceptibility patterns. No significant differences were observed between results obtained by the reference procedures and those achieved by commercial techniques such as the E-test and Sensititre YeastOne.

It should be noted that candins are inactive against *Cryptococcus* species. Candins are cyclic hexapeptides that disrupt the cell wall glucan formation by non-competitive inhibition of (1,3)-β-D-glucan synthase. The drugs lack of activity against *Cryptococcus* species is due to greater proportion of (1,3)-α-D-glucan linkages present in the cell wall polymers of members of this genus [31].

In view of the different susceptibility profiles of fungal species, characterization at species level of clinical isolates of the *Cryptococcus* is compulsory. In addition, the identification of these fungi can be difficult for the clinical laboratories, making it necessary to dispatch isolates of *Cryptococcus* spp. to reference centres. Since such identification could take several weeks, *in vitro* susceptibility testing of isolates belonging to genus *Cryptococcus* should be carried out in clinical laboratories on a routine basis. The importance of the use of these procedures is demonstrated by the fact that antifungal resistance in *Cryptococcus* species might be clinically relevant, as shown by the intrinsic

Table 3 Summary of seven reports from the literature, which presented data on susceptibility profiles of non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species. Ranges of MIC values and Geometric Mean (GM) are displayed when available in mg/l

Reference and publication year	Method	Species (Nb of strain)	Antifungal agent	Range	GM
Kordossis, 1998 (26)	E-test®	*C. albidus* (1)	Amphotericin B	0.25	0.25
			Flucytosine	1.25	1.25
			Fluconazole	4.0	4.0
			Itraconazole	0.50	0.50
		C. laurentii (1)	Amphotericin B	0.5	0.5
			Flucytosine	1.25	1.25
			Fluconazole	4.0	4.0
			Itraconazole	0.50	0.50
Ryder, 1998 (29)	CLSI*	*C. humicola* (1)	Fluconazole	4.0	4.0
García-Martos, 2002 (25)	CLSI	*C. albidus* (14)	Amphotericin B	0.12–1.0	–
			Flucytosine	≥64	–
			Fluconazole	т64.0	–
			Itraconazole	8.0–≥16.0	–
		C. laurentii (4)	Amphotericin B	0.50–1.0	–
			Flucytosine	4.0–16.0	–
			Fluconazole	16.0–32.0	
			Itraconazole	0.25–0.50	–
		C. humicola (5)	Amphotericin B	0.50–1.0	–
			Flucytosine	4.0–16.0	–
			Fluconazole	8.0–16.0	–
			Itraconazole	012–0.50	–
		C. uniguttulatus (7)	Amphotericin B	0,25–1.0	
			Flucytosine	≥64	
			Fluconazole	≥64	
			Itraconazole	0.50–2.0	
		C. luteolus (2)	Amphotericin B	0.12–0.50	
			Flucytosine	≥64	
			Fluconazole	4.0–16.0	
			Itraconazole	0.06.0.25	
	Sensititre®	*C. albidus* (14)	Amphotericin B	0.06–1.0	–
			Flucytosine	≥64	–
			Fluconazole	≥64.0	–
			Itraconazole	2.0–≥16.0	–
		C. laurentii (4)	Amphotericin B	0.50–1.0	–
			Flucytosine	8.0–16.0	–
			Fluconazole	8.0–16.0	
			Itraconazole	0.25–0.50	–
		C. humicola (5)	Amphotericin B	0.50–1.0	–
			Flucytosine	2.0–4.0	–
			Fluconazole	8.0–16.0	–
			Itraconazole	025–0.50	–
		C. uniguttulatus (7)	Amphotericin B	0.25–0.50	
			Flucytosine	≥64	
			Fluconazole	≥64	
			Itraconazole	0.50–2.0	
		C. luteolus (2)	Amphotericin B	0.25.0.50	
			Flucytosine	≥64	
			Fluconazole	8.0–16.0	
			Itraconazole	0.03–0.06	
Serena, 2004 (28)	CLSI	*C. albidus* (10)	Amphotericin B	0.12–2.0	0.42
			Fluconazole	0.50–256.0	9.8
			Itraconazole	0.03–1.0	0.40
			Voriconazole	0.06–32.0	1.2
			Ravuconazole	0.06–32.0	1.2
			Albaconazole	0.06–32.0	0.45
			Micafungin	64.0–128.0	118.3
			Terbinafine	16.0–32.0	29.6

(Continued)

Table 3 (*Continued*)

Reference and publication year	Method	Species (Nb of strain)	Antifungal agent	Range	GM
		C. laurentii (10)	Amphotericin B	0.25–0.5	0.41
			Fluconazole	16–64	35.75
			Itraconazole	0.03–0.25	0.09
			Voriconazole	0.06–0.25	0.10
			Ravuconazole	0.06–0.25	0.08
			Albaconazole	0.03–0.12	0.06
			Micafungin	128.0	128.0
			Terbinafine	8.0–32.0	14.2
Quindós, 2004 (27)	ATB Fungus®	*C. albidus* (5)	Flucytosine	0.25–≥128.0	36.7
		C. laurentii (2)	Flucytosine	0.25	0.25
Shimokawa, 2005 (30)	CLSI	*C. curvatus* (2)	Fluconazole	8.0–16.0	12.0
			Itraconazole	0.50–1.0	0.75
Pedroso, 2006 (16)	CLSI	*C. albidus* (17)	Amphotericin B	0.25–1.0	–
			Flucytosine	0.50–>64.0	–
			Fluconazole	4.0–>64.0	–
			Itraconazole	0.12–8.0	–
		C. laurentii (14)	Amphotericin B	0.12–2.0	–
			Flucytosine	2.0–>64.0	–
			Fluconazole	0.50–32.0	–
			Itraconazole	0.06–1.0	–
		C. uniguttulatus (3)	Amphotericin B	0.25–1.0	–
			Flucytosine	16.0–>64.0	–
			Fluconazole	1.0–>64.0	–
			Itraconazole	0.25–8.0	–
	EUCAST&	*C. albidus* (17)	Amphotericin B	0.25–1.0	–
			Flucytosine	0.50–>64.0	–
			Fluconazole	2.0–>64.0	–
			Itraconazole	0.06–8.0	–
		C. laurentii (14)	Amphotericin B	0.12–2.0	–
			Flucytosine	1.0–>64.0	–
			Fluconazole	1.0–16.0	–
			Itraconazole	0.06–1.0	–
		C. uniguttulatus (3)	Amphotericin B	0.50	–
			Flucytosine	16.0–>64.0	–
			Fluconazole	2.0–>64.0	–
			Itraconazole	0.50–4.0	–

*CLSI: Clinical Laboratory Standards Institute, technique M27-A2. &: European Committee on Antimicrobial Susceptibility testing.

resistance that these species have against candins and the possible development of resistance to azole compounds.

Commercial methods such as E-test and Sensititre YeastOne could be reliable alternatives to detect decreased *in vitro* susceptibility in these species.

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