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In Vitro Antifungal Activity of Isavuconazole against 345 Mucorales Isolates Collected at Study Centers in Eight Countries

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Summary

Although mucormycoses (formerly zygomycoses) are relatively uncommon, they are associated with high mortality and treatment options are limited. Isavuconazole is a novel, water soluble, broad-spectrum azole in clinical development for the treatment of invasive aspergillosis and candidiasis. The objective of this report was to collate data on the *in vitro* activity of isavuconazole against a collection of 345 diverse Mucorales isolates, collected and tested at eight study centers in Europe, Mexico and North America. Each study center undertook minimum inhibitory concentration (MIC) susceptibility testing of their isolates, according to EUCAST or CLSI guidelines. Across all study centers, isavuconazole exhibited MIC_{50} values of 1–4 mg/L and MIC_{90} values of 4–16 mg/L against the five genera. There were also marked differences in MIC distributions, which could be ascribed to differences in inoculum and/or endpoint. EUCAST guidelines appeared to generate modal MICs 2-fold higher than CLSI. These results confirm that isavuconazole possesses at least partial antifungal activity against Mucorales.

Key words: **Isavuconazole, mucorales, mucormycoses, zygomycetes, zygomycosis, EUCAST, CLSI, MIC.**

INTRODUCTION

The traditional phylum Zygomycota (the zygomycetes) includes several genera that are significant in human or animal disease. Of note are the *Absidia*, *Mucor*, *Rhizomucor* and *Rhizopus* genera contained in the order Mucorales, with *Rhizopus* spp. as the most commonly implicated causative organisms of mucormycoses (formerly zygomycoses) in humans [1,2]. According to widespread new proposals, the phylum Zygomycota will no longer exist and the order Mucorales will be reclassified within the subphylum Mucoromycotina [3]. Mucorales are considered to be aggressive and opportunistic pathogens, mainly affecting immunocompromised patients during chemotherapy or following transplantation (particularly hematopoietic stem cell) and patients with type II diabetes mellitus. However, infection can occur occasionally in patients with no known risk factors [4]. Mucormycosis remains relatively uncommon, lagging behind fungal infections caused by *Aspergillus* spp. and *Candida* spp., but its incidence is rising in single institutions that care for a large number of patients with cancer [2,5-8]. They are associated with high mortality and may occur in any body site but mostly as rhinocerebral, pulmonary, cutaneous, gastrointestinal or disseminated zygomycosis. Historically, standard pharmacotherapy has been limited to amphotericin B,[9,10] and although liposomal formulations have improved the renal toxicity profile of this polyene drug,

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clinical experience is scant and the optimal dose remains unclear [11]. Currently, posaconazole is the only marketed azole with activity, albeit less than amphotericin B, against Mucorales [11]. However, posaconazole is not the drug of choice for mucormycosis and its efficacy has only been stated in salvage-therapy studies [12,13]. A recent study has shown that the combination of echinocandins plus amphotericin B may have a role in the treatment of mucormycosis [14]. As a consequence of the relative rarity of mucormycosis and the lack of randomized and prospective clinical trials, the choice of antifungal agents suitable for their treatment has remained limited through the lack of clinical trial data [15].

Isavuconazole is a novel water-soluble azole with a broad spectrum of activity [16] and excellent bioavailability in humans,[17] and is currently in late-stage clinical development for the treatment of invasive aspergillosis and candidiasis. Excellent *in-vitro* activity of isavuconazole against *Candida* spp.,[18-20] *Cryptococcus* spp.,[21] and *Aspergillus* spp.[22] has been described [23]. By contrast, publications relating to the activity of isavuconazole against the Mucorales include relatively few isolates [24–30]. In this paper we collate data relating to Mucorales from independent *in-vitro* studies on isavuconazole performed at eight study centers (*Table 1*).

METHODS

A total of 345 Mucorales isolates, mostly of clinical origin held in culture collections, were collected at the eight study centers in Europe, USA and Mexico (*Table 1*) between 1986 and 2007.

Each study center undertook the susceptibility testing of isolates collected at their institution (*Table 2*). Susceptibility testing at Study Center 1 (Innsbruck, Austria) was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology for spore-forming moulds,[31] and recorded complete growth inhibition at 48 h. In addition, the center also performed MIC testing for isavuconazole against hyphal growth at 48 h and 30°C, using the method of Lass-Flörl *et al.* [32,33]. The remaining seven centers performed susceptibility testing according to the Clinical and Laboratory Standards Institute (CLSI) reference method for filamentous fungi (M38-A) [34]. Growth inhibition of 100% (MIC-0) and 80% (MIC-1) was recorded at 24 h at Study Center 3 [25]. Growth inhibition was additionally recorded at 48 h at Study Center 6. Variations in methodology among study centers are shown in *Table 2*.

Table 2 also shows the criteria defined in the CLSI document M38-A2 [35] for the susceptibility testing of filamentous fungi such as the Mucorales, against a range of antifungal agents including posaconazole and ravuconazole. These antifungal agents are not water-soluble and detailed directions are provided in the CLSI document for the preparation of the dilution series using the appropriate solvent. For uniformity of testing, isavuconazole was also diluted in DMSO.

Study Center 3 also performed MIC testing of isavuconazole using E-test strips (AB Biodisk, Solna, Sweden). The MIC was determined as the drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the E-test strip. To match the scale used in the CLSI procedure, the MIC value read from the E-test strip after 24 h was raised up to the corresponding two-fold dilution as used in CLSI methodology.

The antifungal activity was expressed as the MIC range and MICs at which 90% and 50% of isolates tested were inhibited (MIC_{90} and MIC_{50}, respectively) at 24 h (48 h for Study Center 1).

ANTIFUNGAL AGENTS

All isolates were tested against isavuconazole (ISA), obtained as a reagent-grade powder from Basilea Pharmaceutica Ltd (Basel, Switzerland), and most were also tested against posaconazole (POS) and/or ravuconazole (RAV). Data relating to fluconazole and voriconazole are omitted from this report as the lack of efficacy of these antifungal agents against the Mucorales is well documented [36-38].

RESULTS

The *in vitro* activity of ISA was determined against 345 isolates of Mucorales comprising 80 *Absidia* spp. (49 *A. corymbifera*; 31 unspecified), 18 *Cunninghamella* spp. (3 *C. bertholletiae*; 15 unspecified), 77 *Mucor* spp. (19 *M. circinelloides*; 2 *M. ramosissimus*; 1 *M. rouxianus*; 55 unspecified), 29 *Rhizomucor* spp. (11 *R. pusillus*; 18 unspecified), and 139 *Rhizopus* spp. (28 *R. arrhizus*; 44 *R. microsporus*; 15 *R. oryzae*; 52 unspecified) (*Table 1*).

Across all study centers and following 24 h incubation, ISA exhibited MIC_{50} values of 1 to 4 mg/L and MIC_{90} values of 4 to 16 mg/L against the five genera.

Regardless of the genera tested, there were marked differences in the MIC distributions among the study centers (*Table 3, Figure 1*). At Study Center 6, MICs determined at 48 h were generally higher than those determined at 24 h.

The activity of all comparator compounds was not determined at each study center. POS was co-tested at four study centers and on the basis of MIC_{50}/MIC_{90} values, was approximately 4-fold more active than ISA against each of the Mucorales genera. RAV exhibited similar MICs to ISA.

Study Center 3 also determined ISA MICs using E-test strips. In general, relative to the CLSI method, the E-test tended to generate MICs typically 2- to 4-fold higher at low MIC values but considerably higher at high MICs.

Study Center 1 also determined the MICs of ISA against hyphae and these values were similar to those obtained against the standard conidial suspension.

TABLE 1 - *Source of Mucorales isolates.*

Study Center Number	Study Location	*Absidia*	*Cunninghamella*	*Mucor*	*Rhizomucor*	*Rhizopus*	*Syncephalastrum*	Total	Source of Isolates
1	Innsbruck, Austria	8	3	9	9	7	0	36	From various specimens including blood, respiratory tract, biopsies and other deep body sites; collected from 1996 to 2006, at Innsbruck Medical University, Austria
2	Nuevo León, Mexico	17	0	16	0	27	0	60	Clinical isolates from the Facultad de Medicina, Universidad Autónoma de Nuevo León, Mexico.
3	Madrid, Spain	6	4	21	0	12	2	45	From a collection assembled between 1986 and 2007, predominantly from clinical sources (mainly respiratory or blood), at the Hospital General Universitario Gregorio Marañón, Universidad Complutense de Madrid, Spain.
4	Basel, Switzerland	5	2	3	3	7	0	20	Reference strains from type culture collections at Basilea Pharmaceutica, Basel, Switzerland.
5	Nijmegen, The Netherlands	17	0	13	6	62	0	98	Collected over 15 years and stored at Radboud University Nijmegen Medical Center, The Netherlands
6	Manchester, UK	20	2	3	5	3	0	33	From culture collection at the Regional Mycology Laboratory Manchester (RMLM), UK
7	Ohio, USA	1	0	2	0	2	0	5	From culture collection at the Cleveland Center for Medical Mycology, Ohio, USA
8	Texas, USA	6	7	10	6	19	0	48	From the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, Texas, USA
Total		**80**	**18**	**77**	**29**	**139**	**2**	**345**	

TABLE 2 - *Variations in susceptibility test methodology.*

Study Center	General method	Growth inhibition endpoint	Inoculum size (CFU/mL)	Testing range (mg/L)	Incubation temperature	Control isolates	Notes
1	EUCAST	100% at 48 h, determined visually	1–2.5 x 10^5 (final)	ISA 0.0312–16	37°C	*A. fumigatus* ATCC 204305 (ISA MIC, 1 mg/L; POS MIC 0.25 mg/L); *A. flavus* ATCC 204304 (ISA MIC, 1 mg/L; POS MIC 0.5 mg/L)	Media: RPMI 1640 with 2% glucose
	Hyphal MICs	100% at 24 and 48 h		ISA	30°C for 12–20 h, then 37°C for 12-18 h		Method of Lass-Flörl et al.[32,33]
2	CLSI M38-A	100% at 24 h	1–5 x 10^4 (final)	ISA 0.015–8 POS 0.015–8 RAV 0.015–8	35°C	*C. krusei* ATCC 6258 (ISA MIC, 0.125 mg/L); *C. parapsilosis* ATCC 22019 (ISA MIC, 0.06 mg/L)	Broth macrodilution: RPMI 1640 at pH 7.0.
3	CLSI M38-A	100% (MIC-0) and 80% (MIC-1) at 24 h, read macroscopically	0.4–5 x 10^4 (final)	ISA 0.015–16	35°C	*A. fumigatus* ATCC 204305 (ISA MIC, 0.25–0.5 mg/L); *A. flavus* ATCC 204304 (ISA MIC, 1 mg/L)	
	E-Test	24 h*	2–25 x 10^5 (final)	ISA	35°C		Media: RPMI+2% glucose agar plates
4	CLSI M38-A	80% at 24 h, measured by spectrophotometry at 600 nm	1–3 x 10^3 (final)	ISA 0.0004–100 RAV 0.0004–100 (both species dependent)	35°C	*C. krusei* ATCC 6258 (ISA MIC, 0.085 mg/L); *C. parapsilosis* ATCC 22019 (ISA MIC, 0.02 mg/L)	Broth microdilution: RPMI-MOPS medium solidified with 0.2% low-melting point agarose
5	CLSI M38-A	100%; read at 24 h	0.4–5 x 10^4	ISA 0.06–8 POS 0.06–64	35°C	*C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019	Broth microdilution

TABLE 2 - *Continued.*

Study Center	General method	Growth inhibition endpoint	Inoculum size (CFU/mL)	Testing range (mg/L)	Incubation temperature	Control isolates	Notes
6	CLSI M38-A	90% at 24 and 48 h	1 x 10^4 (final)	ISA 0.015–8; POS 0.015–8; RAV 0.015–8	37°C	*C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019	Media: RPMI 1640-MOPS at pH 7.0 with 2% glucose
7	CLSI M38-A	80% at 24 h	1.5 x 10^4 (final)	ISA 0.03–32	35°C	*C. krusei* ATCC 6258 (ISA MIC, 0.06 mg/L); *C. parapsilosis* ATCC 22019 (ISA MIC, 0.03 mg/L)	
8	CLSI M38-A	100% at 24 h	0.4–5 x 10^4	ISA POS	35°C	*C. parapsilosis* ATCC 22019 (ISA MIC, 0.06 mg/L; POS MIC, 0.125 mg/L)	
Ref	CLSI M38-A2	POS, RAV, ITR: 100% *Rhizopus* spp., read at 21–26 h	0.4–5 x 10^4 (2x final)	POS 0.03–16; RAV 0.03–16	35°C	Select from: *C. krusei* ATCC 6258; *C. parapsilosis* ATCC 22019. *A. flavus* ATCC 204304; *A.fumigatus* ATCC MYA-3627; *A. flavus* ATCC MYA-3631	RPMI-1640-MOPS at pH 7.0±0.1 at 25°C

MOPS – morpholinepropanesulfonic
* - drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. Because E-test strips contain a continuous gradient of drug instead of 2-fold drug dilutions, the MIC endpoint obtained by the E-test was raised to the next two-fold dilution concentration matching the drug dilution on the scale used for the CLSI procedure

TABLE 3 - *MIC values at 24 h, using CLSI M38-A reference method**

Azole / Center	*Absidia*				*Cunninghamella*				*Mucor*				*Rhizomucor*				*Rhizopus*				**Total**			
	n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)		
		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%
Isavuconazole																								
1*	8	4–8	--	--	3	2–>8	--	--	9	4–>8	--	--	9	2–>8	--	--	7	2–>8	--	--	36	2–>8	8	>8
2	17	2–8	4	8	0	--	--	--	16	2–8	4	8	0	--	--	--	27	1–8	2	4	60	1–8	4	8
3**	6	1–16	--	--	4	2–16	--	--	21	<0.015–>16	2	16	0	--	--	--	12	1–16	1	16	45†	<0.015–>16	2	16
4	5	0.06–0.12	--	--	2	0.25–0.5	--	--	3	0.12–>128	--	--	3	0.015–64	--	--	7	0.12–4	--	--	20	0.015–>128	0.25	4
5	17	0.25–8	1	2	0	--	--	--	13	0.5–>16	4	16	6	0.12–4	--	--	62	0.25–>16	1	2	98	0.12–>16	1	4
6	20	0.03–2	0.5	1	2	0.12–1	--	--	3	2–4	--	--	5	0.25–0.5	--	--	3	0.12–1	--	--	33	0.03–4	0.5	2
7	1	0.5	--	--	0	--	--	--	2	0.12–1	--	--	0	--	--	--	2	0.5	--	--	5	0.12–1	0.5	1
8	6	1	--	--	7	1–16	--	--	10	0.5–4	1	2	6	0.5–2	--	--	19	0.12–16	0.25	1	48	0.12–16	1	4
Total	80	0.03–16	1	8	18	0.12–16	2	16	77	<0.015–>128	4	16	29	0.015–64	2	16	139	0.12–32	1	4	345	<0.015–>128	2	8
Posaconazole																								
2	17	1–2	1	2	0	--	--	--	16	0.5–2	1	2	0	--	--	--	27	0.5–2	1	2	60	0.5–2	1	2
5	17	0.06–0.25	0.12	0.12	0	--	--	--	13	0.06–>16	0.25	1	6	0.03–0.5	--	--	62	0.03–>16	0.5	1	98	0.03–>16	0.25	0.5
6	20	0.03–>8	0.12	0.5	2	0.12–0.5	--	--	3	1–4	--	--	5	0.03–0.25	--	--	3	0.12–1	--	--	33	0.03–>8	0.25	1
8	6	0.25–0.5	--	--	7	0.5–1	--	--	10	0.25–0.5	0.5	0.5	6	0.5–1	--	--	19	0.06–32	0.25	0.5	48	0.06–32	0.5	1
Total	60	0.03–16	0.25	1	9	0.12–1	--	--	42	0.06–32	0.5	2	17	0.03–1	0.25	0.5	111	0.03–32	0.5	1	239	0.03–32	0.5	1
Ravuconazole																								
2	17	2–8	4	8	0	--	--	--	16	2–8	4	8	0	--	--	--	27	1–8	2	4	60	1–8	4	8
4	5	0.12–0.5	--	--	2	0.5–1	--	--	3	0.5–>128	--	--	3	0.06–1	--	--	7	0.12–8	--	--	20	0.06–>128	0.25	1
6	20	0.03–1	0.25	1	2	0.12-0.5	--	--	3	2–4	--	--	5	0.015–0.25	--	--	3	0.06–0.5	--	--	34	0.015–4	0.25	1
Total	42	0.03–8	0.5	8	4	0.12–1	--	--	22	0.5–>128	4	8	8	0.015–1	--	--	37	0.06–8	2	4	114	0.015–>128	2	8

Only MIC range given when n<10 isolates
* except Center 1 (EUCAST methodology; MICs at 48 h)
** MIC-0 (100% growth inhibition). Also 2 isolates of *Syncephalastrum* spp.: isavuconazole MICs 0.25 and 8 mg/L
† includes 2 isolates of *Syncephalastrum* spp.

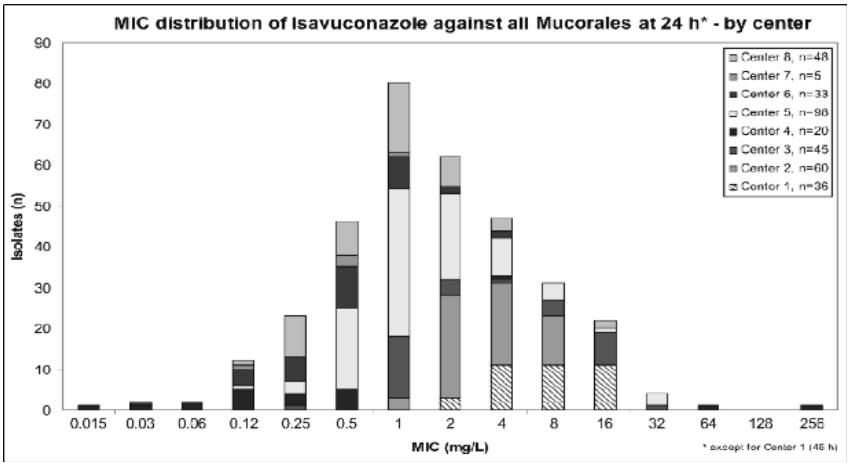

FIGURE 1 - MIC distribution at 24 h for isavuconazole against all Mucorales.

DISCUSSION

Although mucormycoses are relatively uncommon, they are associated with high mortality and current treatment options are limited [9–11]. Among the newer azoles, posaconazole exhibits the best *in vitro* activity against the Mucorales [11]. Hence the primary objective of this study was to assess the *in vitro* activity of isavuconazole, a new azole with potent activity against *Candida* spp. and *Aspergillus* spp., against a large collection of Mucorales. This was achieved by collating the raw data from eight study sites in Europe, USA and Mexico. In this general overview, isavuconazole exhibited at least moderate activity towards Mucorales with MIC_{50} values of 1–4 mg/L and MIC_{90} values of 4–16 mg/L against the five genera.

The susceptibility of isolates was tested using the newly introduced EUCAST guidelines for susceptibility testing of conidia forming moulds [31] at one study center or the CLSI guidelines for susceptibility testing of filamentous fungi [34] at the other seven centers. As is apparent from *Table 2*, the CLSI guidelines allow considerable potential for laboratory to laboratory variation. Consequently a second objective of this study was to investigate the effects of laboratory variation upon MIC determinations.

Four study centers (2, 3, 5 and 8) applied similar CLSI test conditions (inoculum of 0.4–5 x 10^4 CFU/mL, 24-hour incubation and 100% growth inhibition for the MIC determination) and obtained similar MIC distributions, i.e. MIC ranges of 0.12–32 mg/L with modal MICs of 1–2 mg/L. Study Centers 3, 6 and 7 employed an 80% endpoint resulting in MIC ranges of <0.015–16 mg/L with a modal MIC of 0.5 mg/L. Study Center 4 employed the least severe test conditions, i.e. reduced inoculum of 10^3 CFU/mL and 80% endpoint, which resulted in typical MICs of 0.015–0.5 mg/L and a mode MIC of 0.12 mg/L. Interestingly, Study Center 4 also identified the two most isavuconazole-resistant isolates: *Rhizomucor pusillus (*MIC 64 mg/L*)* and *Mucor rouxianus* (MIC 128 mg/L). In contrast, Study Center 1 employed the new EUCAST methodology, which entails a higher inoculum of 10^5 CFU/mL and a 48-hour incubation period, and this resulted in an MIC range of 2–16 mg/L and modal MIC of 4–8 mg/L. MIC results from the E-test were generally much higher than broth dilution methods, suggesting that further development of this diagnostic test is required.

The efficacy of isavuconazole towards filamentous fungi has been investigated in animal infection models. In a neutropenic murine model of disseminated *Aspergillus flavus*, isavuconazole demonstrated impressive antifungal activity in both survival and tissues burden [39]. In this study, mice were treated with isavuconazole pre- (PRE) or post- (POST) infection and treatment continued for a further 10 days. In the PRE models, isavuconazole (6 mg/kg) was 100% protective, itraconazole was 67% protective, but voriconazole (10 mg/kg) had 100% mortality. In the POST models, survival was >66% with isavuconazole (15 and 30 mg/kg dose), similar to voriconazole and itraconazole. In addition, isavuconazole achieved sterilization of all organs in 11/16 survivors.

Despite the laboratory variation in the present study, isavuconazole does possess *in-vitro* activity against all the Mucorales genera tested and might have a role in the treatment and prevention of mucormycosis. Posaconazole usually exhibited somewhat higher *in-vitro* activity and has similar plasma protein binding of >98% in humans. Compared with recent posaconazole clinical experience,[40] the isavuconazole regimen currently in clinical development is expected to result in 2- to 8-fold higher trough levels, consistently higher than 1 mg/L within the first days of treatment. *In-vivo* studies against the Mucorales have not been conducted. However, it will be interesting to investigate isavuconazole's activity and pharmacokinetics in ongoing clinical studies.

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TABLE 4 - *MIC values using alternative methodologies and endpoints.*

	Absidia				***Cunninghamella***				***Mucor***				***Syncephalastrum***				***Rhizopus***				**Total**			
	n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)		
		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%
Centre 3, Isavuconazole																								
Etest 24 h	6	0.25–64	--	--	4	0.5–64	--	--	21	0.25–64	2	64	2	0.25–0.5	--	--	12	0.5–64	1	64	45†	0.25–64	2	64
Etest 48 h	6	1–64	--	--	4	4–64	--	--	21	0.25–64	32	64	2	0.25–4	--	--	12	1–64	64	64	45†	0.25–64	32	64
CLSI MIC-1*	6	0.5–8	--	--	4	0.5–8	--	--	21	<0.015–16	1	8	2	0.125–4	--	--	12	0.5–4	0.5	4	45†	<0.015–16	1	8

	Absidia				***Cunninghamella***				***Mucor***				***Rhizomucor***				***Rhizopus***				**Total**			
	n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)			n	MIC (mg/L)		
		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%		range	50%	90%
Centre 6, CLSI 48 h																								
Isavuconazole	20	0.12–4	0.5	2	2	0.25–2	--	--	3	4	--	--	5	0.25–2	--	--	3	0.12–2	--	--	33	0.12–4	1	4
Itraconazole	20	0.12–16	0.5	>8	2	0.12–0.25	--	--	3	16	--	--	5	0.06–1	--	--	3	0.06–16	--	--	33	0.06–16	0.5	16
Posaconazole	20	0.03–16	0.25	>8	2	0.12–1	--	--	3	1–16	--	--	5	0.12–0.5	--	--	3	0.12–4	--	--	33	0.03–16	0.25	16
Ravuconazole	20	0.12–4	0.25	1	2	0.12–1	--	--	3	4–8	--	--	5	0.12–1	--	--	3	0.06–1	--	--	33	0.06–8	0.5	4
Centre 1, EUCAST, Isavuconazole, Hyphal MICs																								
24 h	8	4	--	--	3	2–4	--	--	9	4	--	--	9	2–4	--	--	7	4	--	--	36	2–4	4	4
48 h	8	>8	--	--	3	>8	--	--	9	>8	--	--	9	4–>8	--	--	7	4–>8	--	--	36	4–16	16	16

Only MIC range given when n<10 isolates
* except Center 1 (EUCAST methodology; MICs at 48 h)
** MIC-0 (100% growth inhibition). Also 2 isolates of *Syncephalastrum* spp.: isavuconazole MICs 0.25 and 8 mg/L
† includes 2 isolates of *Syncephalastrum* spp.

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