Medical Mycology March 2009, **47**, 169–176

informa
healthcare

Susceptibility testing of *Cryptococcus diffluens* against amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole and posaconazole

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Cryptococcus diffluens is a recently re-established species that shares several phenotypic features with *Cryptococcus neoformans.* We evaluated the application of the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) macro- and microbroth dilution methods and the E-test agar diffusion method to determine the *in vitro* susceptibilities of known strains of *C. diffluens* against amphotericin B (AMB), flucytosine (5-FC), fluconazole (FLC), itraconazole (ITC) and the novel triazoles, voriconazole (VRC) and posaconazole (PSC). Seven strains were found to be resistant *in vitro* to AMB (MICs $\geq$2 μg/ml), five were resistant to 5-FC (MICs of $\geq$32 μg/ml), four were resistant to FLC (MICs of FLC $\geq$32 μg/ml) and nine were resistant to ITC (MICs of ITC $>$1 μg/ml). In contrast, VRC and PSC showed good *in vitro* activity against *C. diffluens* strains, even those with elevated MICs to amphotericin B and/or established azoles. Most of the isolates were inhibited by 0.5 μg/ml of both VRC and PSC. A clinical isolate showing phenotypic switching exhibited elevated MICs to both agents, i.e., VRC ($>$16 μg/ml) and PSC ($>$8 μg/ml).

Keywords *Cryptococcus diffluens*, broth dilution methods, agar diffusion method, voriconazole, posaconazole

Introduction

Cryptococcus diffluens is a recently re-established species that is known from only a few strains present in culture collections that were originally recovered from sputum, biopsy, nail or environmental samples [1,2]. Recently, we reported the presence of the species in a case of primary subcutaneous cryptococcosis [3]. The natural niche and geographical distribution of the species is unknown. *C. diffluens* shares several phenotypic characteristics with *Cryptococus neoformans* and its identification requires sequence analysis of parts of the rRNA genes [3]. However, these techniques are not routinely used in clinical laboratories for cryptococcal identification. Therefore, it seems likely that *C. diffluens* remains largly underdiagnosed among clinical isolates. Previously, we used the CLSI M27-A2 (Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) macrodilution method to determine the *in vitro* susceptibilities of *C. diffluens* strains to amphotericin B (AMB), flucytosine (5-FC), fluconazole (FLC), itraconazole (ITC), ketoconazole, miconazole and terbinafine [3]. Voriconazole (VRC) and posaconazole (PSC) are two new triazole antifungal agents with broad spectrum activity against opportunistic fungi, including *C. neoformans* [4–11]. The agar based E-test procedure was suggested as a reliable technique for *in vitro* susceptibility testing of *C.neoformans* to VRC and PSC [12], AMB, FLC, ITC [13] and 5-FC [14]. The

Received 21 October 2007; Final revision received 27 April 2008; Accepted 19 May 2008

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DOI: 10.1080/13693780802213407

aim of the present study was to evaluate the use of three different methods, i.e., macro- and microbroth dilution and an agar diffusion to determine the *in vitro* susceptibility of *C.diffluens* against AMB, 5-FC, FLC, ITC, VRC and PSC.

Materials and methods

Isolates

Eleven *C. diffluens* strains preserved in the collection of the CBS were used for the tests [3]. We included the CLSI recommended quality control strains (QCs) *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258, and the reference strains *C. neoformans* ATCC 90112 and *Candida albicans* ATCC 90028. Prior to testing, each isolate was twice subcultured onto fresh Sabouraud Dextrose agar (SDA) to ensure optimal growth capabilities.

Antifungal susceptibility tests

Each isolate was tested by the CLSI reference macrodilution method and by two microdilution methods [15], as well as the agar diffusion technique using E-test. QC and reference strains were tested each time along with *C. diffluens* isolates.

The broth dilution tests

AMB (Bristol-Meyers Squibb, Wallingford, Conn.), 5-FC (Sigma Chemical Co., St. Louis, Mo.), FLC (Pfizer, Istanbul, Turkey), and ITC (Janssen Pharmaceuticals, Beerse, Belgium), were obtained as standard antifungal powders. VRC (Pfizer, Istanbul, Turkey) and PSC (Schering-Plough, Istanbul, Turkey) were obtained from the respective manufacturers as defined powder. Stock solutions were prepared in water (FLC, 5-FC), dimethyl sulfoxide (AMB, VRC) and polyethylene glycol (ITC, PSC). Serial two fold dilutions were prepared as outlined in the CLSI M27-A2 reference broth macro- and microdilution method [15]. The final concentrations of the antifungal agents ranged from 0.03 to 16 μg/ml for AMB and ITC, 0.125 to 64 μg/ml for FLC and 5-FC, 0.015 to 16 μg/ml for VRC and 0.015 to 8 μg/ml for PSC. In a previous study [3], we performed preliminary investigations to search for the optimal test conditions including incubation time and temperature, time of reading and nutrient medium**.** In the present study, as per the results previously obtained [3], the broth macrodilution and microdilution tests were performed in accordance with the guidelines in CLSI document M27-A2 [15]. Morpholinepropanesulfonic acid (0.05 mol/liter; MOPS, Sigma) buffered yeast nitrogen base medium (YNB; Difco) (pH 5.4 for 5-FC and pH 7.0 for the remaining antifungals) supplemented with 2% glucose was used as test medium [3,15,16]. Inoculum suspensions were each prepared from five colonies of five day-old cultures grown on SDA at 30°C. Cell suspensions prepared in 5 ml of sterile (0.85%) saline, were vortexed for 15 seconds and the turbidity of the suspensions adjusted to 0.5 McFarland turbidity standard at 530 nm wavelength. These were further diluted 1:100 and then 1:20 for the macrobroth procedure, and 1:50 and then 1:20 for the microbroth procedure in test medium to obtain a final inoculum concentration of approximately 5.0×10^2–2.5×10^3 CFU ml^{-1} for macrobroth and 0.5×10^3 to 2.5×10^3 CFU ml^{-1} for the microbroth tests. In this study, additional preliminary experiments were performed twice to determine the growth patterns of individual *C. diffluens* strains in liquid media to aid in the reading of results from the broth dilution tests with both tubes containing 1 ml and wells of microplates containing 100 μl of each final inoculum suspension and incubated at 35°C. Yeast growth was also examined microscopically by India ink preparations. Drug-free and yeast-free controls were included in the studies. Microbroth dilution tests were performed in two parallel series of 96-well microtitre plates (each isolate was tested by two parallel microdilution methods). Test tubes and microplates were incubated at 35°C. MICs were determined visually at the first 24 h interval when growth was observed in the drug-free control tube or well. In macrodilution tests the MIC endpoints were determined according to CLSI specifications of 80% reduction in turbidity compared with that of the drug-free growth control for azoles and 5-FC, and complete inhibition of growth for AMB. Using the microdilution technique, MIC endpoints were read with the aid of a reading mirror. In one set of microdilution plates, the MIC endpoints were read according to the M27-A2 guidelines; the MICs for azoles and 5-FC were defined as the first sharp or obvious decrease in growth (50% inhibition) and complete inhibition of growth for AMB. In the second set of microdilution plates, the MIC was determined by a colorimetric method utilizing an oxidation-reduction indicator. The tetrazolium salt 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) was used for colorimetric MIC reading. XTT is a yellow tetrazolium salt that is converted by mitochondrial dehydrogenases of viable (i.e., metabolically active) yeast cells to an orange formazan product [17–19]. XTT (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline at concentration of 0.25 mg/ml and 50 μl of XTT was added to all wells when

growth was observed in the drug-free control tube or well. The plates were incubated for 3 h at 35°C to allow for color development. This color change was assessed visually. The MIC in the XTT assay was determined visually as the lowest concentration of antifungal agent at which no color change occured. Experiments were repeated twice on different days.

Although interpretive guidelines and breakpoints for susceptibility testing of *C. neoformans* are not yet available from CLSI, it has been suggested that isolates for which MICs are ≥ 2 μg/ml should be regarded resistant to AMB [20] and those with MICs to 5-FC of ≥ 32 μg/ml as resistant and isolates with MICs 8–16 μg/ml to 5-FC as intermediate [14,15,21]. A break point for susceptibility of ≤ 1 μg/ml for both VRC and PSC was used in previous studies [4,6,15,22]. In an earlier investigation [6], the interpretive criteria for susceptibility to FLC and ITC were those published by the CLSI [15]. For FLC, strains with MICs of ≤ 8 μg/ml were considered susceptible; susceptible dose dependent at 16 to 32 μg/ml and resistant with MICs of ≥ 32 μg/ml. In the case of ITC, the cut off for susceptible was ≤ 0.125 μg/ml, susceptible dose dependent at 0.25 to 0.5 μg/ml and resistant at ≥ 1μg/ml. In this study, these definitions were adopted for AMB, 5-FC, FLC, ITC, VRC and PSC.

Agar diffusion test

Preliminary tests. Owing to different growth features of *C. diffluens* observed in response to various conditions, preliminary experiments were performed in a previous study [3] to search an adequate medium and incubation time using four solidified (1.5% agar) and buffered (pH 5.4 and 7.0 ± 1) media, i.e., yeast nitrogen base agar (YNBA), Antibiotic Medium 3 (AM3) (Difco Laboratories, Detroit, MI, USA), RPMI 1640 medium, and Mueller Hinton agar (BBL, Becton, Dickinson and Company, Sparks, MD, USA veya Le Pont de Claix, France) supplemented with 2% glucose [15,23]. Eleven *C. diffluens*, QC and reference *Candida* and *Cryptococcus* isolates were included. The agar plates were inoculated twice by dipping a sterile cotton swab into the appropriate inoculum suspension adjusted spectrophotometrically to the turbidity of No.1 and No. 2 McFarland standards and streaking it across the entire surface of the agar in three directions prior to incubation at 30° and 35°C. Dalmou plate cultures were done on YNBA as growth control by another inoculation technique using a high inoculum concentration, i.e., a loopful from a yeast colony, at the same temperatures. Growth was monitored from 24 h to 2 weeks to determine the optimal incubation temperature and time of reading on agar media. To maintain adequate moisture, agar plates were sealed with parafilm. Preliminary tests were performed in duplicate.

The E-test procedure. The antifungal agents evaluated were AMB, 5-FC, ITC, VRC, PSC (concentration range 0.002 to 32μg/ml), and FLC (concentration range 0.016 to 256 μg/ml). The E-test method was performed according to the manufacturer's guidelines using YNBA at pH 5.4 for 5-FC and pH 7.0 for the remaining antifungals buffered with 0.165 M MOPS [14,15]. The inoculum suspensions of *C. diffluens* isolates matched the turbidity of no.1 McFarland standard. The yeast inoculum was swabbed onto the surface of the agar plate twice and was allowed to dry for 15 min before the addition of the E-test strip. One E-test strip was placed on each plate. The inverted plates were incubated at 30°C and read when the lawn of growth was clearly visible. The MICs by the E-test was the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. Tests were performed in duplicate.

Analyses of susceptibility results. All susceptibility tests were repeated twice for each method. Essential agreement (EA) was defined as MIC results of AMB, 5-FC, FLC, ITC, VRC and PSC obtained by either microbroth dilution test or the XTT assay and the reference macrobroth method were in agreement or within two dilutions. Because the E-test strips contain a continuous gradient of each drug tested instead of the $\log_2$ drug dilution scheme of the broth macrodilution method, the E-test MICs were elevated to the next drug concentration that matched the macrodilution scheme to facilitate comparison of results [24]. However, no MIC value was obtained by this technique as is mentioned in the results.

Major errors were defined as results in which the reference macrodilution method result was susceptible and the reference microdilution method or XTT assay result was resistant, while very major errors were defined as results in which the reference method result was resistant and the microdilution method or XTT assay result was susceptible [17].

Results

Growth patterns in liquid media

The growth patterns of *C. diffluens* in broth media were strain specific. CBS 926, CBS 965, CBS 986 grew as sediment, CBS 160 and CBS 1925 were flocculent and

accumulated in the lower part of the tube, CBS 10266 grew as a pellice to slightly turbid and the remaining strains were turbid. In macrodilution tests, good shaking of each tube prior to reading aided to an accurate definition of the density, i.e., the growth. However, strain specific nonhomogenous growth of *C. diffluens* strains complicated the determination of MICs and did not allow for precise quantification of fungal growth in small scale tests within the relatively small amounts of liquid media present in the wells of microplates. Small amount of pseudohyphae-like encapsulated cells were observed in clinical isolate CBS 10266 in india ink stained slides (Fig. 1).

Growth conditions on solidified media

No visible colony growth was obtained from cell suspensions adjusted to No. 1 and No. 2 McFarland standards on all cultures of solid media at 30°C and 35°C. Visible colonies appeared within five to six days at 30°C and relatively poor growth was obtained within eight days at 35°C on Dalmau plate cultures inoculated with a high concentration of (i.e., a loopfull from a colony) of yeasts. QC and reference *Candida* strains grew at 24 h on all of the agar media.

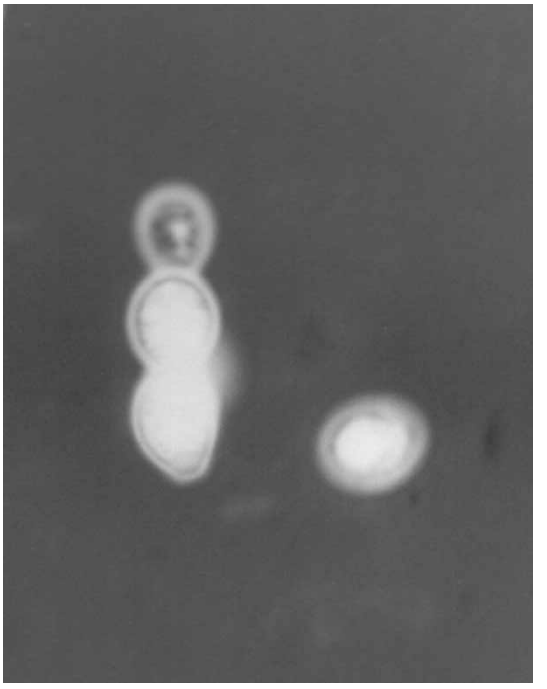

Fig. 1 Pseudohyphae-like encapsulated cells produced in liquid media were observed in clinical isolate CBS 10266 (India ink preparation, ×40).

Susceptibility to antifungals

Growth was obtained within 24 to 48 h in macro- and microbroth dilution tests. No growth was obtained with the E-test, which is in agreement with our findings on solid media. The *in vitro* susceptibilities of 11 *C. diffluens* isolates to AMB, 5-FC, FLC, ITC, VRC and PSC, as determined by the CLSI macro- and microdilution method are shown in Table 1.

With the reference macrodilution test, three isolates of *C. diffluens* were found to be susceptible to AMB at MIC of ≤ 1 μg/ml, seven were resistant with MICs of ≥ 2 μg/ml. Five isolates were resistant to 5-FC exhibiting MICs of ≥ 32 μg/ml, seven were susceptible to FLC at MICs of ≤ 8 μg/ml. Four strains were resistant against FLC at MICs of ≥ 32 μg/ml, while two strains were susceptible to ITC at MIC of ≤ 0.125 μg/ml and nine others were resistant at MIC of ≥ 1 μg/ml. Ten isolates were susceptible at MICs of ≤ 1 μg/ml of VRC and nine were susceptible at MICs of ≤ 1 μg/ml of PSC. *C. diffluens* strain CBS 2824 showed an elevated MIC value against PSC, but a low MIC against VRC. Isolate CBS 10266 showed high MIC values to both antifungal agents. None of the isolates was found to be fully susceptible to all azoles tested and no FLC resistance was found without resistance to ITC. CBS 2824 showed cross resistance to FLC and PSC and CBS 10266 showed resistance to both of the new azoles, VRC and PSC. In general the MIC values for each duplicate test were identical. In addition, MIC values for quality control strains included in the experiments agreed with those published in the CLSI document [15].

The overall EA was 100% (11/11) for all antifungal agents tested with MICs measured by macrodilution and microdilution reading with XTT reading technique. Using readings according to the CLSI instructions, the EAs were 100% (11/11) for AMB and FLC, 90% (10/11) for VRC and 82% (9/11) for 5-FC, ITC and PSC. Discrepancies between the broth dilution test methods were observed usually within one two-fold dilutions. However, very major errors or major errors did not occur between macrobroth and microbroth dilution MICs. With the agar diffusion method, all strains appeared as susceptible due to lack of growth on solidified media.

Discussion

In vitro susceptibility testing of *C. neoformans* may be performed using either broth-based or agar-based methods [6]. At present, the CLSI microbroth dilution method in microplates is widely employed for research purposes. Previous evaluations of the E-test method

Table 1 Susceptibilities of molecularly identified 11 *Cryptococcus diffluens* to AMB, 5-FC, FLC, ITCI VRZ and PSZ determined by CLSI methodologies and utilizing XTT reading technique.

Strains	Method/MIC reading technique		MIC (μg/ml)					
			AMB	5-FC	FLC	ITC	VRZ	PSZ
CBS 160	Macrobroth dilution		16	0.5	64	16	0.5	0.125
	Microbroth dilution	CLSI	8	0.125	32	4	0.25	0.3
		XTT	16	0.5	64	16	0.5	0.125
CBS 926	Macrobroth dilution		4	0.25	4	16	0.25	0.06
	Microbroth dilution	CLSI	2	0.6	2	8	0.25	0.03
		XTT	4	0.25	4	16	0.25	0.06
CBS 966	Macrobroth dilution		2	1	4	4	0.125	0.015
	Microbroth dilution	CLSI	1	1	2	1	0.6	0.015
		XTT	2	1	4	4	0.125	0.015
CBS 6436	Macrobroth dilution		0.03	0.5	>64	16	0.06	0.03
	Microbroth dilution	CLSI	0.03	0.5	32	16	0.3	0.015
		XTT	0.03	0.5	>64	16	0.06	0.03
CBS 964	Macrobroth dilution		8	0.5	4	16	0.06	0.25
	Microbroth dilution	CLSI	4	0.5	4	8	0.06	0.125
		XTT	8	0.5	4	16	0.06	0.25
CBS 965	Macrobroth dilution		0.03	0.5	1	8	0.015	0.125
	Microbroth dilution	CLSI	0.03	0.25	1	4	<0.015	0.3
		XTT	0.03	0.5	1	8	0.015	0.125
CBS 986	Macrobroth dilution		1	32	0.5	16	2	0.06
	Microbroth dilution	CLSI	0.5	8	0.25	8	0.5	0.03
		XTT	1	32	0.5	16	1	0.06
CBS 1925	Macrobroth dilution		16	64	<0.125	16	0.015	0.06
	Microbroth dilution	CLSI	8	32	0.125	8	<0.015	0.03
		XTT	8	64	<0.125	16	0.015	0.06
CBS 1929	Macrobroth dilution		16	64	64	4	0.25	0.5
	Microbroth dilution	CLSI	8	64	32	4	0.125	0.25
		XTT	16	64	64	4	0.25	0.5
CBS 2824	Macrobroth dilution		16	64	64	<0.03	0.25	>8
	Microbroth dilution	CLSI	8	64	16	0.03	0.125	
		XTT	16	64	16	<0.03	0.25	>8
CBS 10266	Macrobroth dilution		16	32	4	0.5	>16	>8
	Microbroth dilution	CLSI	8	64	4	1	>16	>8
		XTT	16	32	4	0.5	>16	>8

have indicated that this agar-based method is a reliable and easy-to-use alternative to the CLSI reference method for routine purposes with *C. neoformans* [25]. In the present study, the CLSI macro- and microbroth dilution methods and an agar diffusion method, viz., E-test were evaluated for determining the susceptibility of *C. diffluens* isolates against AMB, 5-FC, FLC, ITC, VRC and PSC. Reagarding the previously observed fastidious growth of *C. diffluens* strains in response to different incubation temperatures and various agar media [3], two groups of preliminary experiments were performed to determine (i) growth patterns in broth medium and (ii) the optimal growth medium, incubation temperature and time of reading the E-test. Further, microdilution MICs were determined by two different techniques, i.e., using CLSI specifications and the XTT reading technique.

In liquid media, the growth of the homogenous unicellular yeasts may be determined by visual inspection by grading turbidity or by spectrophotometric, turbidimetric or nephalometric analysis. These detection methods are based on the scattering of radiation by a solution containing solid particles and accurate determination may be associated with the homogenous dispersion of the particles in the suspension. The spectrophotometric approach to MIC endpoint determination provides for objective and rapid reading and eliminates the subjective judgements concerning minimal turbidity and trailing that confound antifungal susceptibility testing [26,27]. For *Candida* species,

studies by Anaissie *et al*. [28] have shown that the visual determination of microdilution MIC endpoints may be aided by agitation of microdilution trays prior to reading of the MICs. Such agitation disperses the unicellular yeast cells within the wells, producing a homogenous suspension, and minimizes the effects of trailing that plague the interpretation of azole susceptibility tests. Two to three small cells adhered as catenulate pseudohyphae-like growth patterns and this non-homogenous development of *C. diffluens* strains may hamper the determination of MICs and prevent precise quantification of fungal growth. Previous studies [29] have shown that the accuracy of spectrophotometric readings may be negatively impacted by clumps of mycelial-like growth. Alternatively, biomass can be determined colorimetrically using indicator substances that are reduced to colored products by viable microorganisms. Thus, fungal biomass is estimated by the metabolic activity of the fungus [17]. In our experiments MIC reading utilizing the XTT technique gave results in agreement with the reference macrodilution method.

Several previous reports have noted that the E-test is a reliable alternative, poor growth of *C. neoformans* by E-test has been previously reported and is in agreement with our findings [30,31].

Definitive diagnosis of cryptococcal infection is confirmed by the culture of specimens, often cerebrospinal fluid or blood. Even cryptococcal antigen positivity in cerebrospinal fluid or blood may be taken as a presumptive diagnosis of cryptococcosis. However, *C. diffluens* shares several phenotypic characteristics with *C. neoformans*, the main aetiological agent of cryptococcosis, including capsule antigen factors [3]. Therapeutic management of cryptococcal infections usually consists of primary therapy with AMB, with or without 5FC, followed by maintenance therapy with FLC [32–34]. However, both acquired [6,35,36] and intrinsic *in vitro* [34,37] resistances to commonly used antifungal agents AMB, 5FC, and FLC occur among *C. neoformans* isolates. This may be partly attributable to the incidental presence of phenotypically similar non-*C. neoformans Cryptococcus* strains among clinical isolates.

Strains CBS 6436, CBS 1929, and CBS 2824 that exhibited high *in vitro* MICs against FLC, but were found to be susceptible to both VRC and PSC showing low MICs ($\leq$0.05 μg/ml). These agents were reported by several authors to demonstrate good *in vitro* activity against *C. neoformans* [5–11]. In a recent study covering a large number of isolates, both VRC and PSC were found to be even more active than FLC against *C. neoformans* [4]. Although factors other than drug susceptibility (e.g., host's immune status, site of involvement) have been accounted for the success of antifungal therapy [38], our data suggest alternative agents of therapy against cryptococcal infections.

C. diffluens strain CBS 10266, which showed phenotypical switching and originated from a case of primary subcutaneous cryptococcosis of an otherwise healthy young man, showed high MICs in duplicate tests to both triazoles VRC and PSC. It was demonstrated that phenotypic switching, which is associated with changes in the polysaccharide capsule and the cell wall in *C. neoformans*, may effect the outcome of infection [39]. Moreover, it was suggested that antifungal interventions may select for the more virulent mucoid variant, which could affect the outcome of infection in chronically infected hosts [40–42]. The relevance of phenotypic switching in infection with *C. diffluens* and response to treatment is still unknown. However, it has been suggested that selection of switching variants is promoted in the setting of antifungal therapy. Interestingly, in our previous study [3], this isolate showed a relatively low *in vitro* MIC value (4 μg/ml) to FLC and even lower MIC values to ITC, KTC, and MCZ. In comparison to AMB, FLC, and ITC [3], both VRC and PSC had the lowest MIC for *C. diffluens.* Both agents inhibited the majority of *C. diffluens* isolates, including FLC-resistant ones, at an MIC of $\leq$1 μg/ml.

Interpretative breakpoints have not been assigned to VRC and PSC for *Cryptococcus* species. However, preliminary pharmacokinetic data indicated that levels of these agents achievable in serum may range from 2 to 6 μg/ml depending on the dose regimen [43–45]. Therefore, we used a break point for susceptibility of $\leq$1 μg/ml for both agents [4,6]. In our study, VRC and PSC had fungistatic activity beyond safely achievable concentrations in serum for all, except two isolates. Both antifungal agents seem promising agents for the treatment of infections with this cryptococcal species.

In conclusion, CLSI reference broth dilution methods may be used for the accurate testing of antifungal susceptibility of non-*neoformans Cryptococcus* isolates. As shown here, *C. diffluens* isolates were susceptible to both VRC and PSC. Importantly, both of the new triazoles remained active against most strains of *C. diffluens*, including those with elevated MICs to AMB and/or FLC.

Acknowledgements

We thank Pfizer, Inc and Schering Plough for supplying VRZ and PSZ antifungal powders.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 24 July 2008.