Eur J Clin Microbiol Infect Dis (2016) 35:1803–1810
DOI 10.1007/s10096-016-2731-8

ORIGINAL ARTICLE

Molecular characterisation and antifungal susceptibility of clinical *Cryptococcus deuterogattii* (AFLP6/VGII) isolates from Southern Brazil

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Received: 3 June 2016 / Accepted: 11 July 2016 / Published online: 1 August 2016

Abstract Cryptococcosis, caused by *Cryptococcus gattii sensu lato*, is an emerging disease that was initially found in (sub)tropical regions but recently expanded to temperate regions. *Cryptococcus gattii s.l.* infections are mostly encountered in healthy individuals, frequently affecting both lungs and the central nervous system (CNS). Usually, *C. gattii s.l.* is less susceptible to antifungal compounds than its counterpart, *C. neoformans s.l.* We studied 18 clinical *C. gattii s.l.* isolates with amplified fragment length polymorphism (AFLP) fingerprinting, mating-typing, multi-locus sequence typing (MLST) and antifungal susceptibility testing. All isolates were *C. deuterogattii* (genotype AFLP6/VGII), 14 were mating-type α and four were type a. Amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole showed high activity, with minimum inhibitory concentration (MIC) ranges of 0.063–0.25, 0.031–0.25, 0.031–0.25, 0.031–0.25 and <0.016–0.25 μg mL^{-1}, respectively. Fluconazole and flucytosine had high geometric mean MICs of 2.07 and 3.7 μg mL^{-1}, respectively. Most cases occurred in immunocompetent patients ($n = 10$; 55.6 %) and CNS involvement was the most common clinical presentation ($n = 14$; 77.8 %). Three patients (16.7 %) showed sequelae, hyperreflexia, dysarthria, diadochokinesia, anosmia and upper limb weakness. In conclusion, all infections were caused by *C. deuterogattii* (AFLP6/VGII) and the majority of patients were immunocompetent, with the CNS as the most affected site. All antifungal drugs had high in vitro activity against *C. deuterogattii* isolates, except fluconazole and flucytosine.

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Introduction

Cryptococcosis is caused by basidiomycetous yeast species that belong to the *Cryptococcus gattii*/*Cryptococcus neoformans* species complexes, mainly affecting lungs and the central nervous system (CNS) [1]. The taxonomy of the polyphyletic genus *Cryptococcus* has been thoroughly revised [2–4]. The two varieties of *C. neoformans* were recognised as species, with *C. neoformans* (=formerly *C. neoformans* variety *grubii*) and *C. deneoformans* (=formerly *C. neoformans* variety *neoformans*) [2]. The five *C. gattii* species complex genotypes were raised to the species level as *C. gattii sensu stricto* (AFLP4/VGI), *C. bacillisporus* (AFLP5/VGIII), *C. deuterogattii* (AFLP6/VGII), *C. tetragattii* (AFLP7/VGIV) and *C. decagattii* (AFLP10/VGIV) [2]. *Cryptococcus gattii s.s.*

and *C. deuterogattii* are the main culprits of infections in immunocompetent hosts [2, 5], whereas *C. bacillisporus*, *C. tetragattii* and *C. decagattii* are commonly associated with immunocompromised hosts [2, 6–8].

Members of the *C. gattii* species complex have increasingly been reported from temperate climate regions [5, 9–11]. Trading of tree products, transport of propagules through water currents and via animals, insects and humans may be responsible for the world-wide spread [12, 13]. The first environmental isolation of *C. gattii s.l.* was reported from *Eucalyptus camaldulensis* [14]. Globally, *C. gattii s.l.* has been found on a plethora of other species of trees [13, 15–19].

In Europe, *C. gattii s.s.* is the most frequently encountered species, while few cases of *C. deuterogattii* infection are travel-related [20–22]. In Asia and Australia, *C. gattii s. s.* and *C. deuterogattii* are predominant [23]. *Cryptococcus bacillisporus* is rarely encountered outside North and South America [21, 23, 24], while *C. tetragattii* has mainly been isolated from Africa and India [8, 23]. On the American continent, *C. deuterogattii* is the predominant species among clinical and environmental isolates [23]. In North America, *C. deuterogattii* subgenotypes (AFLP6A/VGIIa, AFLP6B/VGIIb and AFLP6C/VGIIc) were reported as the cause of expanding outbreaks [5, 9, 10, 25]. A recent study showed that, by using coalescence gene genealogy analysis, the ancestral lineage of *C. deuterogattii* originated from South America, specifically from the Brazilian Amazon Rainforest, where mating-types a and α were reported in nearly equal rates, providing evidence of recombination within the Brazilian *C. deuterogattii* population [25].

Cryptococcus gattii s.l. is less susceptible to common antifungal compounds than *C. neoformans*; fluconazole and flucytosine show lower in vitro activity against *C. deuterogattii* strains [26–28]. In addition, heteroresistance to azoles in the *C. neoformans*/*C. gattii* species complexes is an intrinsic mechanism that may contribute to relapse of cryptococcosis during maintenance therapy [29, 30].

In the present study, our aim was to compare the clinical outcome with molecular and antifungal susceptibility data of Brazilian *C. gattii s.l.* isolates.

Materials and methods

Isolates and clinical data

Eighteen clinical *C. gattii s.l.* isolates were isolated at the Hospital de Clínicas, Curitiba, PR, Brazil between 1999 and 2015. A single colony was taken for further microbiological and molecular characterisation. Medical records were accessed to collect clinical information.

Molecular characterisation

Extraction of genomic DNA, mating-type determination, amplified fragment length polymorphism (AFLP) fingerprinting and multi-locus sequence typing (MLST) were performed as described previously [20–22, 31].

Sequences were compared with those deposited in the MLST database (http://mlst.mycologylab.org). The alignment was performed with the online MAFFT alignment module [32] and visual inspection by MEGA version 7 [33], followed by a 1000× bootstrapped maximum likelihood analysis on an MLST dataset that comprises all known *C. deuterogattii* sequence types [8].

Antifungal susceptibility testing

Broth microdilution testing [34] included amphotericin B (Bristol Myers Squibb, Munich, Germany), fluconazole (Pfizer, Sandwich, United Kingdom), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), voriconazole (Pfizer), posaconazole (Merck, Kenilworth, NJ, USA), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland) and flucytosine (ICN Pharmaceuticals, Zoetermeer, The Netherlands). The concentration ranges were 0.016–16 μg mL^{-1} for amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole, and 0.062–64 μg mL^{-1} for fluconazole and flucytosine. *Cryptococcus* isolates were cultured onto Sabouraud dextrose agar for 48 h at 30 °C and the inocula were adjusted to 1×10^3 CFU/mL in 0.9 % NaCl to perform the test. The microdilution plates were incubated at 35 °C for 72 h and the minimal inhibitory concentrations (MICs) were defined as the lowest concentration that produced complete growth inhibition for amphotericin B and prominent decrease of growth (50 %) for other antifungal agents when compared with the drug-free growth control. *Candida parapsilosis* ATCC22019 and *C. krusei* ATCC6258 were used as quality controls [34]. The interpretation of MIC values was based on the epidemiological cut-off value (ECV) [26–28]. MIC_{50} and MIC_{90} values were obtained by ordering the data for each antifungal in ascending order and selecting the median and 90th quantile, respectively. Geometric mean MICs were calculated using Microsoft Office Excel 2010 software (Microsoft, Redmond, WA, USA). When the MIC was higher or less than the dilutions tested, 1 $\log_2$ dilution higher or 1 $\log_2$ dilution lower was used to calculate the geometric mean.

Results

Molecular characterisation

Mating-type analysis showed that 14 isolates were mating-type α and four were type a. MLST analysis showed that all 18

isolates clustered with the *C. deuterogattii* AFLP6/VGII reference strain WM178 (Fig. 1). Maximum likelihood analysis showed that most isolates were related with other Brazilian isolates, while isolate UFU986 was genetically indistinguishable from the outbreak genotype AFLP6A/VGIIa (Fig. 1). MLST sequences were deposited in GenBank with the accession numbers KU642658–KU642671, KU642673–KU642676 (*CAP59*), KU642703–KU642716, KU642718–KU642721 (*GPD1*), KU642748–KU642761, KU642763–KU642766 (IGS1), KU642793–KU642806, KU642808–KU642811 (*LAC1*), KU642838–KU642851, KU642853–KU642856 (*PLB1*), KU642883–KU642896, KU642898–KU642901 (*SOD1*) and KU642928–KU642938, KU642940–KU652944, KU642946, KU642966 (*URA5*). By AFLP genotyping analysis, four isolates clustered together with the *C. deuterogattii* AFLP6/VGII reference strain WM178, while the remaining isolates clustered in two distinct clades due to the presence of several dominant markers (Fig. 2).

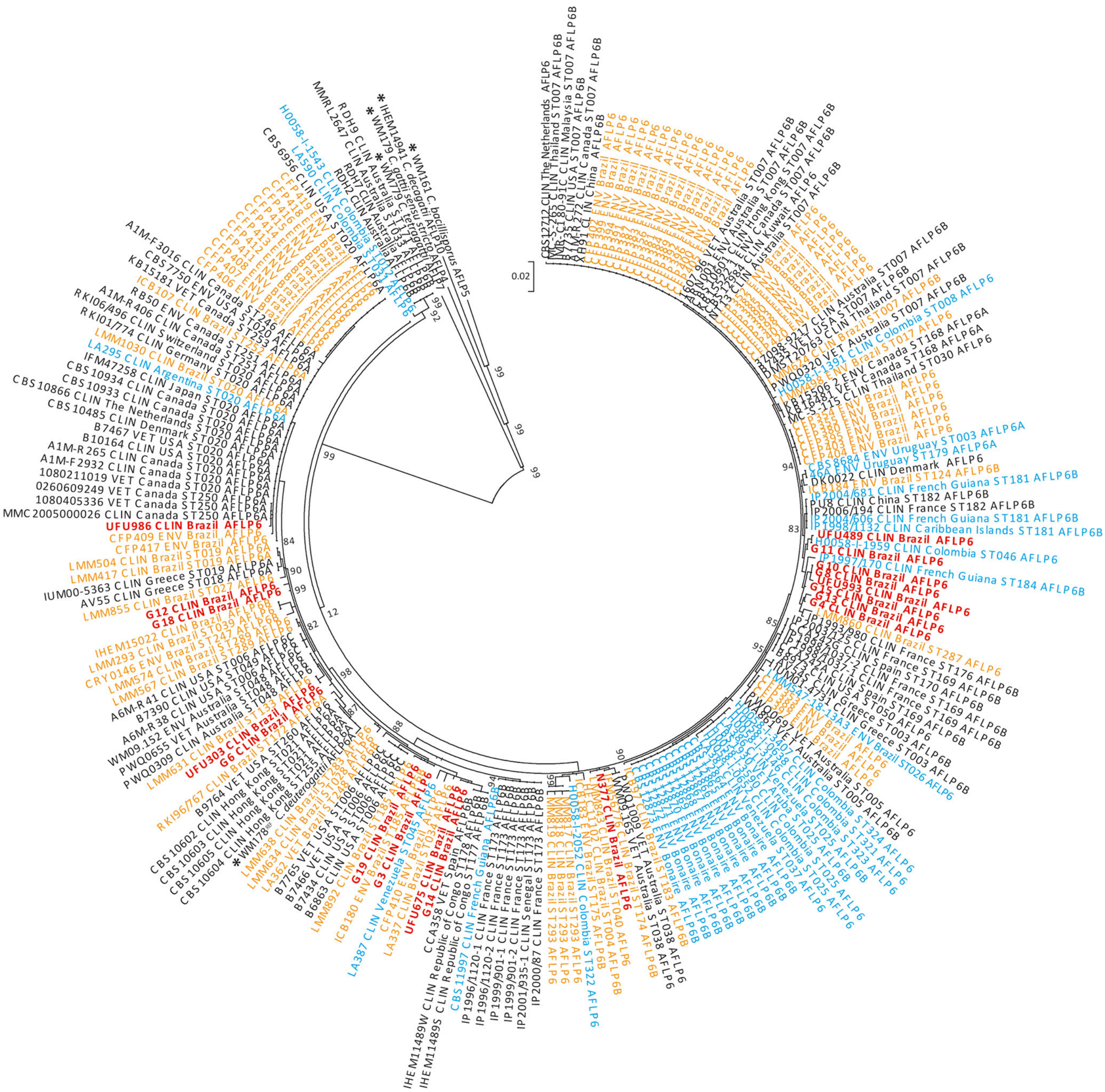
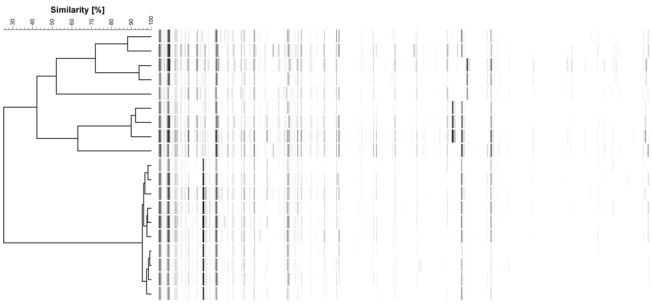


Fig. 1 Phylogenetic 1000× bootstrapped maximum likelihood analysis of *Cryptococcus deuterogattii* isolates based on the ISHAM consensus MLST dataset. *Cryptococcus gattii sensu stricto* (WM179), *C. bacillisporus* (WM161), *C. tetragattii* (WM779) and *C. decagattii* (IHEM14941) were used as an outgroup. Bold red, isolates from the present study; orange, other Brazilian isolates; blue, other Latin American isolates; black, global isolates; *, reference strains



Isolate#	Mating-type	Sex	Age (y)	Occupation	Immune status	Source	Treatment	Outcome	City (State)	Year
UFU993	a	M	72	Farmer	Immunocompetent	Blood/Skin	AMB (1) / FLC (400)	Died	Uberlândia (MG)	2014
WM178^{REF}	-	-	-	-	-	-	-	-	-	-
UFU303	alpha	F	30	Housewife	Immunocompetent	CNS	AMB (1) / FLC (400)	Cured	Uberlândia (MG)	2015
UFU986	alpha	M	55	-	Immunocompetent	CNS	AMB (1)	Died	Uberlândia (MG)	2008
G6	alpha	M	-	-	-	Lungs	-	-	-	2005
G18	alpha	F	20	Supermarket employee	Immunocompetent	CNS/Lungs	ISA (200)	Cured / Sequelae	Almirante Tamandaré (PR)	2012
G3	alpha	M	51	Seller	Immunocompetent	CNS/Lungs	AMB (1) / ITC (100)	Died	Curitiba (PR)	2002
G12	a	M	-	-	-	CNS	-	-	Curitiba (PR)	2009
G4	alpha	M	76	-	-	Skin	-	-	Curitiba (PR)	2005
G10	alpha	F	-	-	Immunocompetent	CNS	-	-	Curitiba (PR)	2009
N377	alpha	F	45	Housekeeper	HIV-infected	CNS	AMB (1) / FLC (400)	Died	Curitiba (PR)	2006
G13	alpha	M	60	Retired	Immunocompetent	CNS	AMB (1) / FLC (400)	Died	Colombo (PR)	2010
G14	a	M	32	Lumberjack	Immunocompetent	CNS/Lungs	AMB (3) / FLC (800)	Cured / Sequelae	Almirante Tamandaré (PR)	2010
G19	alpha	F	35	Farmacist	Immunocompetent	CNS/Lungs	ISA (200)	Cured	São José dos Pinhais (PR)	2013
UFU489	alpha	F	31	Housewife	HIV-infected	CNS	AMB (1) / FLC (400)	Cured	Uberlândia (MG)	1999
G15	alpha	M	48	Retired	HIV-infected	CNS	AMB (1) / FLC (800)	Cured	Curitiba (PR)	2011
UFU675	a	M	50	-	HIV-infected	CNS	AMB (1) / FLC (400)	Died	Uberlândia (MG)	2007
G11	alpha	M	38	Mason	Immunocompetent	CNS/Lungs	AMB (3) / FLC (400)	Cured / Sequelae	Pinhais (PR)	2009
G8	alpha	-	-	-	-	Blood	-	-	Curitiba (PR)	2008

Fig. 2 Amplified fragment length polymorphism (AFLP) fingerprint patterns of 18 Brazilian *C. deuterogattii* (AFLP6/VGII) isolates, including clinical and epidemiological characteristics of the patients. WM178 (*C. deuterogattii* AFLP6/VGII) serves as a reference strain; AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; ISA, isavuconazole; treatment doses, AMB (mg/kg/day) and FLC, ITC and ISA (mg/day); –, data not available; F, female; M, male; CNS, central nervous system. PR, Paraná State; MG, Minas Gerais State

Antifungal susceptibility profiles

The MIC values are presented in Table 1. Itraconazole, voriconazole, posaconazole, isavuconazole and amphotericin B showed high activity against *C. deuterogattii*. Fluconazole and flucytosine had the highest geometric mean MICs of 2.07 and 3.7 μg mL^{-1}, respectively.

Clinical data

The majority of isolates were recovered from immunocompetent patients ($n = 10$; 55.6 %). Fourteen (77.8 %) had CNS involvement and four (22.2 %) of them were HIV-positive. Furthermore, six patients (33.3 %) had lung infection, including five (27.7 %) with associated CNS symptoms, while two patients (11.1 %) had cutaneous cryptococcosis. Three (16.7 %)

Table 1 Minimal inhibitory concentrations (MICs) of all the *Cryptococcus deuterogattii* isolates studied

Isolate code	Minimal inhibitory concentration (μg mL^{-1})						
	AMB	FLC	ITC	VOR	POS	ISA	5FC
G3	0.063	0.5	0.063	0.063	0.063	0.031	4
G4	0.125	8	0.25	0.25	0.25	0.25	8
G6	0.031	8	0.125	0.125	0.125	0.063	2
G8	0.031	2	0.125	0.063	0.063	0.063	4
G10	0.125	4	0.125	0.125	0.125	0.125	8
G11	0.063	0.5	0.031	0.031	0.031	<0.016	1
G12	0.063	0.5	0.063	0.063	0.063	0.063	4
G13	0.031	4	0.063	0.063	0.063	0.063	2
G14	0.063	4	0.125	0.125	0.125	0.125	4
G15	0.125	4	0.125	0.125	0.125	0.125	4
G18	0.125	2	0.125	0.063	0.063	0.063	4
G19	0.125	16	0.25	0.25	0.25	0.25	4
N377	0.125	0.5	0.063	0.063	0.063	0.031	8
UFU303	0.125	8	0.125	0.125	0.125	0.063	4
UFU489	0.063	0.5	0.063	0.063	0.063	0.031	2
UFU675	0.125	0.5	0.063	0.063	0.063	0.063	4
UFU986	0.063	4	0.25	0.125	0.125	0.125	4
UFU993	0.125	1	0.063	0.063	0.063	0.063	4
MIC range	0.063–0.25	0.5–16	0.031–0.25	0.031–0.25	0.031–0.25	<0.016–0.25	1–8
MIC_{50}	0.063	2	0.125	0.063	0.063	0.063	4
MIC_{90}	0.125	8	0.25	0.125	0.125	0.125	8
Geometric mean	0.0788	2.0785	0.0994	0.0853	0.0853	0.0651	3.7034

AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; ISA, isavuconazole; 5FC, flucytosine

developed neurological sequelae after the cryptococcal infection, one left-sided hyperreflexia, dysarthria and diadochokinesia, one had motor sequelae in the upper limbs and one developed anosmia. Nine patients (50 %) were treated with a combination of amphotericin B and fluconazole, one received amphotericin B and itraconazole, one amphotericin B and two were treated with isavuconazole. Two patients received liposomal amphotericin B after development of nephrotoxicity. Treatment dosing and outcome data are summarised in Fig. 2.

Discussion

Infections caused by members of the *C. gattii* species complex have a wide spectrum of clinical presentations, including pneumonia, meningoencephalitis, skin lesions and pulmonary and cerebral cryptococcoma formation, and surgical intervention may be necessary in certain cases [6, 35–38]. CNS disease is associated with neurological sequelae such as visual impairment, deafness, limb weakness and dysphasia [39]. We observed hyperreflexia, dysarthria, diadochokinesia, anosmia and upper limb weakness. Some studies reported that 60–90 % of patients with *C. gattii* species complex cryptococcosis have pulmonary symptoms with or without CNS involvement [10, 39, 40]. These data differ from this study, where the majority of patients ($n = 14$; 77.8 %) had CNS disease, five (27.7 %) also had lung involvement and a solitary patient (5.5 %) had only a lung infection.

All isolates were found to be *C. deuterogattii*, which is in concordance with the literature data, as it is predominant among clinical, veterinary and environmental isolates in Brazil [18, 41–50]. *Cryptococcus gattii s.s.* is more often reported in southern Brazil [51, 52] and the southeastern region [52, 53], and *C. bacillisporus* in the southeastern and northeastern part [52]. Despite increasing epidemiological surveys in Brazil, information about the species distribution are fragmented and underrepresented from several Brazilian states. This is mainly due to the lack of proper diagnosis and the absence of an efficient reporting system [43].

We found that four out of 18 *C. deuterogattii* were mating-type a. The presence of both mating types within the South American *C. deuterogattii* population demonstrates that recombination events are a common phenomenon, causing high genetic diversity compared to other localities [25]. Globally, the majority of clinical isolates were found to be mating-type α, and with respect to *C. deuterogattii*, this is also the case for the ongoing outbreaks in North America [6, 25]. Recently, the first Australian *C. deuterogattii* mating-type a isolate was reported [54]. The absence or low number of mating-type a isolates within study populations suggests that *C. deuterogattii* is either clonal or reproduces by same-sex mating [55].

Antifungal susceptibility testing showed that amphotericin B had high activity, which is in concordance with other studies [27, 48, 56–58]. Fluconazole had the lowest activity among the triazoles tested, with an MIC range of 0.5–16 μg mL^{-1}. Nevertheless, all MIC values were in the susceptible range [26, 59]. Hagen et al. [58] observed lower fluconazole activity against all *C. gattii* species complex members, with European and North American isolates showing higher MIC values (0.125 to >64 μg mL^{-1} and 0.5–64 μg mL^{-1}, respectively). A recent Brazilian study reported high MICs (2–64 μg mL^{-1}), with a geometric mean of 6.08 μg mL^{-1} for *C. deuterogattii* [60]. We also observed low MICs of itraconazole and new triazoles [26, 27, 56, 58].

Isavuconazole is a second-generation triazole antifungal, with a broad spectrum of activity against many important fungal pathogens [61]. Antifungal susceptibility tests have shown potent in vitro activity against members of *C. neoformans*/*C. gattii* species complexes, even for those that are less susceptible to fluconazole [28, 62, 63]. We observed that isavuconazole had excellent activity against *C. deuterogattii*, showing the lowest geometric mean MIC (0.065 μg mL^{-1}) among the antifungal compounds tested. Two immunocompetent female patients were included in a multi-centre international clinical trial with isavuconazole (ClinicalTrials.gov, NCT00634049) [64]. The first was 20 years old and working in a supermarket, where she had continuous contact with wood, fruits and vegetables. After the diagnosis, she received amphotericin B deoxycholate therapy for 16 days, but without response. The therapy was switched to isavuconazole, resulting in a complete clinical, radiological and microbiological response after 6 months of treatment. Anosmia was observed as a sequel. The second patient was a pharmacist, who received isavuconazole as primary therapy for a total of 176 days, resulting in a complete response without any sequelae.

Flucytosine had the lowest in vitro activity, showing the highest geometric mean MIC (3.7 μg mL^{-1}) among the antifungals tested, but the MICs for all isolates were within the wild-type range. It is known that resistance to flucytosine is rare [58, 65], but the use as monotherapy could lead to acquired resistance [66]. There are studies showing less susceptibility to flucytosine in *Cryptococcus* [58, 67, 68], but since flucytosine is not registered in Brazil, we supposed that the patients did not receive previous treatment with this drug. Higher MICs of flucytosine were also reported in other Brazilian studies [45, 48, 53, 60].

In conclusion, the majority of cases occurred in immunocompetent patients, with the CNS as the most affected site. All infections were caused by *C. deuterogattii* (AFLP6/VGII) and all antifungal drugs had in vitro activity against this species, except fluconazole and flucytosine.

Acknowledgments The work of Patricia Fernanda Herkert was financially supported by 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior' and 'Conselho Nacional de Desenvolvimento Científico e Tecnológico', Brazil.

Compliance with ethical standards

Conflict of interest JFM served as a consultant to and has received research grants from Astellas, Basilea, Gilead Sciences and Merck. All of the other authors declare no conflicts of interest.

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