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Original Article

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Comparative antifungal susceptibility analyses of *Cryptococcus neoformans* VNI and *Cryptococcus gattii* VGII from the Brazilian Amazon Region by the Etest, Vitek 2, and the Clinical and Laboratory Standards Institute broth microdilution methods

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Abstract

Early diagnosis, efficient clinical support, and proper antifungal therapy are essential to reduce death and sequels caused by cryptococcosis. The emergence of resistance to the antifungal drugs commonly used for cryptococcosis treatment is an important issue of concern. Thus, the *in vitro* antifungal susceptibility of clinical strains from northern Brazil, including *C. neoformans* VNI ($n = 62$) and *C. gattii* VGII ($n = 37$), to amphotericin B (AMB), 5-flucytosine, fluconazole, voriconazole, and itraconazole was evaluated using the Etest and Vitek 2 systems and the standardized broth microdilution (CLSI-BMD) methodology. According to the CLSI-BMD, the most active *in vitro* azole was voriconazole (*C. neoformans* VNI modal MIC of 0.06 μg/ml and *C. gattii* VGII modal MIC of 0.25 μg/ml), and fluconazole was the least active (modal MIC of 4 μg/ml for both fungi). Modal MICs for amphotericin B were 1 μg/ml for both fungi. In general, good essential agreement (EA) values were observed between the methods. However, AMB presented the lowest EA between CLSI-BMD and Etest for *C. neoformans* VNI and *C. gattii* VGII (1.6% and 2.56%, respectively, $P < .05$ for both). Considering the proposed *Cryptococcus* spp. epidemiological cutoff values, more than 97% of the studied isolates were categorized as wild-type for the azoles. However, the high frequency of *C. neoformans* VNI isolates in the population described here that displayed non-wild-type susceptibility to AMB is noteworthy. Epidemiological surveillance of the antifungal resistance of cryptococcal strains is relevant due to the potential burden and the high lethality of cryptococcal meningitis in the Amazon region.

Key words: *in vitro* antifungal susceptibility testing, *C. neoformans*, *C. gattii*, Amazon region.

Introduction

Cryptococcosis is a systemic mycosis affecting humans and animals, which is caused by the yeasts of *Cryptococcus neoformans* sensu latu (containing *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*)[1] and *Cryptococcus gattii s.l.*[2] *C. neoformans* contains the serotypes A and D, and

the major molecular types: VNI/AFLP1, VNB/AFLP1A/AFLP1B, VNII/AFLP1A/AFLP1B, VNIII/AFLP3, and VNIV/AFLP2.[3–5] *C. gattii* contains the serotypes B and C, with the molecular types: VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, and VGIV/AFLP7.[3,5] Since 1980, infection due to the *C. neoformans* in immunocompromised individuals, mainly human immunodeficiency virus (HIV)-infected hosts, have become an important public health problem.[6] The latest estimates suggest that HIV-associated cryptococcal meningitis accounts for 150 000–200 000 deaths per year and is responsible for 15% (95% confidence interval [CI]: 10%–19%) of AIDS-related deaths.[7] Moreover, infections due to the *C. gattii* in apparently immunocompetent hosts are no longer geographically restricted, emerging in the last 30 years in temperate climates, such as the ongoing outbreaks in Canada and the United States Pacific Northwest.[8,9]

C. gattii VGII infections occur in all Brazilian regions, including the Amazon (North) and the Savanna (Northeast).[10,11] Recent studies have reinforced northern Brazil as the most likely origin of *C. gattii* VGII and its subsequent global spread.[12–14] Cryptococcosis caused by *C. gattii* VGII is endemic in northern and northeastern Brazil and is also the major primary cause of meningitis in non-HIV-infected patients in these regions. *C. neoformans* VNI is also endemic in these regions, mostly presenting as meningitis in HIV-infected patients, but it can also occur in individuals without predisposing factors.[10,11]

Irrespective of the causative species, cryptococcal infection of the central nervous system is associated with high mortality rates.[15] Early diagnosis, efficient clinical support, and proper antifungal therapy are essential to reduce death and other negative impacts caused by cryptococcosis. The gold-standard antifungal therapy for cryptococcal meningitis is the combination of amphotericin B deoxycholate (AmB-D; 0.7–1.0 mg/kg per day) and flucytosine (100 mg/kg per day in four divided doses) for the initial 2 weeks, followed by fluconazole (400–800 mg per day for 8 weeks and 200 mg per day thereafter) for a minimum of 1 year and until immune reconstitution.[16]

Fluconazole-resistant *Cryptococcus* isolates have been increasingly reported in cases of therapy failure, mainly associated with AIDS.[17–20] Several factors influence the susceptibility profile of cryptococcal agents to antifungal drugs, such as the use of long-lasting fluconazole suppressive monotherapy, low bioavailability of the drug in the infected tissue, the fungistatic action of azoles, and intrinsic resistance, such as the observed heteroresistance of *C. neoformans* and *C. gattii* to azoles,[21] a potential mechanism that may also be implicated in treatment failures.[22]

The purpose of this study was to determinate the antifungal susceptibility profiles of clinical strains of *C. neoformans* VNI and *C. gattii* VGII from the Brazilian Amazon region to amphotericin B, flucytosine, itraconazole, fluconazole, and voriconazole. The reference method (CLSI M27-A3 broth microdilution (CLSI-BMD)) and two commercially available methods (the Vitek 2 system and the Etest) were compared. The expression of virulence factors, including melanin synthesis, capsule induction, thermotolerance and the production of extracellular phospholipase, were also evaluated for quality control of the strains analyzed.

Methods

Strains

Sixty-two isolates of *C. neoformans* and 37 isolates of *C. gattii s.l.* from 99 different patients from the Amazon region in northern Brazil were included (Supplementary Table 1). All strains were preserved by freeze-drying or by cryopreservation in glycerol at −70°C for a period of 5 to 11 years (1995–2006) in the Culture Collection of Pathogenic Fungi of the National Institute of Infectious Diseases (INI) and in the Culture Collection of Fungi of the National Institute of Quality Control in Health (INCQS) at Oswaldo Cruz Foundation (FIOCRUZ). The strains were recovered and inoculated onto Petri dishes containing yeast extract and malt extract agar (YMA, Difco Laboratories, Detroit, MI, USA) and Niger seed agar (*Guizotia abyssinica*; NSA) to ensure viability and purity, respectively. Each strain was subcultured at least twice onto YMA. All strains were identified by observing colony and cellular morphology and by physiological and biochemical tests.[23]

Reference strains for quality control were derived from international reference culture collections: *C. neoformans* ATCC 32045, ATCC 90112, and ATCC 90113; *C. gattii* ATCC 56990; *Papiliotrema laurentii* ATCC 18803; *Naganishia albida* ATCC 10666; *Saitozyma podzolica* CBS 7717; *Candida parapsilosis* ATCC 22019; and *Candida krusei* ATCC 6258.

Virulence factors

Melanin production was observed by the presence of dark brown colonies[23] after inoculating the strains onto NSA and observing the plates incubated at 25°C for 5 days. The strains *P. laurentii* ATCC 18803, *N. albida* ATCC 10666, and *S. podzolica* CBS 7717 that do not produce melanin were used as negative controls for the NSA medium. *C. neoformans* ATCC 32045 and *C. gattii* ATCC 56990 were used as positive controls. To induce capsule production, the strains were treated according to the methods described by Zaragoza et al.[24] Thermotolerance was evaluated as described previously.[23,25] Extracellular phospholipase production was tested on 2% Sabouraud dextrose agar (SDA, Difco Laboratories, USA) supplemented with 8% egg yolk,[26] using two known strong phospholipase producer strains, *C. neoformans* ATCC 90112 and ATCC 90113, as positive controls.

Genotype determination by *URA5*-RFLP

The DNA of all isolates was extracted as previously described.[27] The molecular types were determined by *URA5*-RFLP analysis of the *URA5* gene. In brief, polymerase chain reaction (PCR)

was performed in a final volume of 50 μl. Each reaction contained 50 ng of DNA, 1 × PCR buffer (200 mM Tris-HCl (pH 8.4)), 500 mM KCl (Invitrogen Co., Waltham, MA, USA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen Co., USA), 2 mM magnesium chloride, 1.5 U Taq DNA polymerase (Invitrogen Co., USA), and 50 ng of each primer (URA5, 5′ ATGTCCTCCCAAGCCCTCGACTCCG 3′ and SJ01, 5′' TTAAGACCTCTGAACACCGTACTC 3′). PCR was performed in an Eppendorf gradient mastercycler (Hamburg, Germany), with a 2 min initial DNA denaturation at 94°C, followed by 35 cycles of 45 s denaturation at 94°C, 1 min annealing at 61°C, 2 min extension at 72°C, and a final extension for 10 min at 72°C. A total of 30 μl of the PCR products was double digested with *Sau*96I (10 U/μl) and *Hba*I (20 U/μl) for 3 h, and the fragments were separated by 3% agarose gel electrophoresis at 100 V. RFLP patterns were assigned visually by comparison with patterns obtained from standard strains (*C. neoformans* VNI-VNIV and *C. gattii* VGI-VGIV).[28]

Broth microdilution antifungal susceptibility testing

BMD was performed in accordance with the recommendations of the CLSI in the M27-A3 protocol[29] (CLSI, 2008). In brief, all the antifungal agents were obtained as reagent grade powders with high purity. Powders were supplied by the manufacturer Sigma Co., USA. The three azoles, itraconazole (ICZ), fluconazole (FCZ) and voriconazole (VCZ), as well as amphotericin B (AMB) and flucytosine (5FC), were diluted according the CLSI protocol. The medium used in the assays was Roswell Park Memorial Institute (RPMI) 1640 (with L-glutamine, without sodium bicarbonate, with phenol red; Invitrogen Co., USA) supplemented with glucose to a final concentration of 2% and buffered with 165 mM MOPS pH 7.0 (Sigma Co., USA). Tests were performed in 96-well, sterile, flat-bottom microplates with lids. Inoculated microplates were incubated at 35°C for 72 h without shaking. Minimum inhibitory concentrations (MICs) were determined spectrophotometrically in a microplate reader with the Microwell System (Organo Teknika, The Netherlands) at a wavelength of 492 nm. Antifungal MICs were defined as the lowest concentration able to inhibit the growth of the microorganism tested in comparison to the growth of the control without any antifungal drugs. This allowed for a quantitative assessment of growth for each isolate in the test. For azoles and 5FC, MICs were read as the lowest concentration of the agent that inhibited growth by 50% compared to growth of the control. For AMB, the MIC was the lowest concentration of drug that completely inhibited growth compared to the growth of the control. The reference strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were used as internal quality controls in all test plates. This antifungal susceptibility testing was repeated at least twice at different times for all 99 strains to check the stability of the strains and the reproducibility of the MIC values obtained.

Vitek 2 compact

The commercial, fully automated method for *in vitro* antifungal susceptibility testing, Vitek 2 (bioMérieux, France), was also used. The AST-YSO1 card is indicated by the manufacturer for the MIC determination of yeast and contains the antifungals AMB, 5FC, FCZ, and VCZ. The steps to perform susceptibility testing include the preparation of an inoculum suspension, inoculation of the AST-YSO1 card, and reading and interpreting the results, all of which were performed in accordance with the manufacturer's instructions in the device's user manual.[30] Tests were performed in duplicate when results of the automated system were discordant to those obtained by the broth microdilution.

Etest

The Etest (bioMérieux, Sweden) was carried out following the manufacturer's guidelines.[31] The medium used for the Etest was RPMI 1640 (with L-glutamine, without sodium bicarbonate; Sigma Co., USA) supplemented with glucose to a final concentration of 2%, 1.5% agar, and buffered with 165 mM MOPS pH 7.0. Petri dishes of 90 mm in diameter containing the RPMI 1640 medium to a depth of 4.0 mm were used. Periodic evaluations for Etest controls were performed with *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and *C. albicans* ATCC 90028, which were used in all tests performed. The plates were inoculated and incubated at 35°C for 48 h. Etest was also carried out at least twice for each strain.

Interpretation of the results

Epidemiological cutoff values (ECVs) are the minimal inhibitory concentration/minimal effective concentration values that separate fungal populations into those with and those without acquired and/or mutational resistance based on their phenotypes (MICs). The ECV MIC values proposed for *C. neoformans* VNI are as follows: AMB 0.5 μg/ml, 5FC 8 μg/ml, FCZ 8 μg/ml, ICZ 0.25 μg/ml, and VCZ 0.25 μg/ml. For *C. gattii* VGII, the ECVs are as follows: AMB 1 μg/ml, 5FC 16 μg/ml, FCZ 32 μg/ml, ICZ 0.5 μg/ml, and VCZ 0.25 μg/ml.[32,33] Strains with MIC values lower or equal to the ECVs were classified as wild-type (WT), and those with MIC values higher than the ECVs were classified as non-wild-type (n-WT).

Data analysis

MIC ranges, MIC_{50}, MIC_{90}, mode, and geometric mean (GM) were calculated for the three methods tested. Moreover, essential agreement (EA) and categorical agreement (CA) between the

CLSI reference method and Vitek 2 and Etest were analyzed. The essential agreement (EA) between the methods was defined as when the MIC results were within ± two dilution ranges. Categorical agreement (CA) was defined as when the two methods agreed with respect to the MIC result categories. The CA was defined as the percentage of isolates classified in the same category (WT or n-WT) using the broth microdilution reference method.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2003. Comparisons between the MIC values obtained by the CLSI-BMD Etest and Vitek 2 were carried out by applying the Wilcoxon test for paired data at a significance level of 0.05. This method considers the matching of the results for each of the isolates of *C. neoformans* VNI and *C. gattii* VGII. The Kolmogorov-Smirnov test was applied to the pair *Cryptococcus neoformans* VNI / *Cryptococcus gattii* VGII to compare MIC values of the five antifungal drugs as determined by the CLSI-BMD gold standard method. The results with *P*-values <.05 in the statistical test indicated significant differences.

Results

Virulence factors

All 99 isolates preserved by freeze-drying or cryopreservation produced melanin, as observed by the production of dark brown colonies on NSA. All isolates also produced capsules ranging from 1.6 to 6.4 μm thick, as observed after induction in specific capsule-inducing medium. All isolates studied grew at 25°C and 36°C after 72 h of incubation. All strains produced extracellular phospholipase that was classified as having moderate or high enzymatic activity, as observed on egg-yolk supplemented SDA.

Genotyping

The *URA5*-RFLP analyses classified the 99 strains included in this study into two groups: the 62 isolates of *C. neoformans* were identified as genotype VNI, and the 37 isolates of *C. gattii* were identified as genotype VGII.

Quality control of antifungal susceptibility tests

To assess the quality of the materials and the correct execution of the tests, quality control strains with known MICs were employed in all tests. The MICs for the CLSI-recommended reference strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were in the respective reference ranges in all procedures. For the Etest, the reference strain, *C. albicans*. ATCC 90028, was also used and its MIC value was within the given reference values (Supplementary Table 2).

CLSI-BMD, Etest, and Vitek 2 susceptibility results

As described above, MIC values obtained by the CLSI-BMD and Etest were obtained after 72 and 48 h, respectively. The interval time required for the Vitek 2 system to obtain MIC results did not differ for *C. neoformans* VNI or *C. gattii* VGII (15.25–27.15 h and 15–27.50 h, respectively). MIC values of the *C. neoformans* VNI and *C. gattii* VGII isolates obtained by each method, expressed as an MIC range, MIC_{50}, Mode, MIC_{90}, and GM values, are summarized in Table 1. The Kolmogorov-Smirnov test, at a significance level of .05, was applied to the pair *Cryptococcus neoformans* VNI / *Cryptococcus gattii* VGII to 5FC, AMB, FCZ, VCZ, and ICZ MIC results as determined by the gold-standard method (CLSI-BMD). Only FCZ and ICZ showed significant differences between the two species (*P* values of 5.5e−05 and 1.728e−08, respectively).

Comparison of CLSI-BMD with Etest and Vitek 2

Essential agreement

The EA among the MIC results of CLSI-BMD compared to the Etest and Vitek 2 system was determined when the MIC results were within a two-dilution range. The EA observed between the MIC values of the CLSI-BMD and Etest for *C. neoformans* VNI and *C. gattii* VGII are summarized in Table 2. In brief, the best EA between these two methods was observed for VCZ and *C. neoformans* and for all azoles and *C. gattii*. AMB presented the lowest EA for *C. neoformans* VNI and *C. gattii* VGII. The EAs between the CLSI-BMD and Vitek 2 MIC values for *C. neoformans* VNI and *C. gattii* VGII are depicted in Table 3. *P*-values for 5FC and FCZ for *C. neoformans* VNI and 5FC and VCZ for *C. gattii* VGII were not determined because all of the MIC values were the same for the Vitek 2 system and CLSI-BMD. Between these two methods, AMB presented the highest EA, whereas 5FC presented the lowest EA.

The EA between CLSI-BMD and the Vitek 2 system for *C. neoformans* VNI and VCZ was not determined because all strains presented MICs that were ≤0.125 μg/ml in the Vitek 2 system, which hindered the comparison with the CLSI-BMD results. The same scenario occurred with 48 strains when analysed for 5FC susceptibility and in 24 strains when analysed for FCZ susceptibility, all of which presented MICs of ≤1 μg/ml. A similar scenario was also observed with *C. gattii* VGII, in which MICs of ≤0.125 μg/ml for VCZ were observed in the Vitek 2 system for 36 strains. One *C. gattii* VGII isolate presented MICs of 0.5 μg/ml and 0.25 μg/ml when VCZ susceptibility was evaluated by the CLSI-BMD and the Vitek 2 system, respectively, indicating an EA in this strain. Additionally, 33 *C. gattii* VGII strains tested for 5FC, and 4 tested for FCZ susceptibility presented MICs of ≤1 μg/ml and therefore were excluded from the EA calculation.

Table 1. *In vitro* susceptibility of *Cryptococcus neoformans* VNI and *Cryptococcus gattii* VGII clinical strains to five antifungal agents as determined by CLSI broth microdilution, the Vitek 2 system and Etest methods.

Species (n° of isolates)	Antifungal agent	Methods	MIC (μg/ml)				
			Range	MIC_{50}	Mode	MIC_{90}	Geometric Mean (95% CI)
C. neoformans VNI ($n = 62$)	Amphotericin B	CLSI	0.5–2	1	1	1	0.925 (0.903–1.024)
		Etest	0.012–0.25	0.094	0.094/0.125	0.125	0.081 (0.081–0.102)
		Vitek 2	1–2	1	1	1	1.011 (1.00–1.048)
	Flucytosine	CLSI	1–8	2	2/4	4	2.735 (2.677–3.387)
		Etest	0.125– > 32	4	4	8	3.789 (3.698–5.978)
		Vitek 2	≤ 1–2	≤ 1	≤ 1	2	NA
	Itraconazole	CLSI	0.06–0.5	0.125	0.125	0.125	0.122 (0.118–0.150)
		Etest	0.032–1	0.125	0.125	0.75	0.154 (0.129–0.334)
	Fluconazole	CLSI	2–8	4	4	4	3.498 (3.419–4.065)
		Etest	2–32	4	8	16	5.967 (5.919–8.565)
		Vitek 2	≤ 1–2	1	2	2	NA
	Voriconazole	CLSI	0.015–0.25	0.06	0.06	0.06	0.049 (0.048–0.068)
		Etest	0.016–0.38	0.047	0.06	0.094	0.047 (0.047–0.070)
		Vitek 2	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125	NA
C. gattii VGII ($n = 37$)	Amphotericin B	CLSI	0.5–2	1	1	1	1.038 (0.986–1.162)
		Etest	0.023–0.38	0.094	0.094	0.125	0.091 (0.085–0.123)
		Vitek 2	0.5–4	1	1	1	1.084 (0.946–1.324)
	Flucytosine	CLSI	0.5–16	4	4	8	2.964 (2.865–4.702)
		Etest	0.125–6	1	0.5	4	1.114 (1.114–1.926)
		Vitek 2	≤ 1–2	≤ 1	≤ 1	≤ 1	NA
	Itraconazole	CLSI	0.125–0.5	0.25	0.25	0.5	0.236 (0.223–0.307)
		Etest	0.047–1.5	0.5	0.5/1	1	0.337 (0.329–0.650)
	Fluconazole	CLSI	2–32	8	4	16	6.758 (6.541–10.432)
		Etest	2–48	8	6/16	24	10.503 (10.216–18.512)
		Vitek 2	≤ 1–32	8	2	16	4.00 (3.818–7.939)
	Voriconazole	CLSI	0.03–0.5	0.25	0.25	0.25	0.174 (0.172–0.230)
		Etest	0.023–0.5	0.094	0.06	0.38	0.119 (0.111–3.231)
		Vitek 2	≤ 0.125–0.25	≤ 0.125	≤ 0.125	≤ 0.125	NA

CI, confidence intervals; MIC, minimum inhibitory concentrations; NA, not applied.
MIC_{50} and MIC_{90}, MICs at which 50% and 90% of isolates were inhibited.

Categorical agreement

The MIC values obtained for *C. neoformans* VNI and *C. gattii* VGII for the CLSI and Etest methods were categorized as wild-type (WT) or non-wild-type (n-WT) according to the ECVs proposed by Espinel-Ingroff[32,33] (Table 4). Excellent CA was observed for comparisons between CLSI and Vitek 2, with the exception of AMB and *C. neoformans* VNI (85.5%). A higher variation in CA was observed between CLSI and the Etest for *C. neoformans* VNI. The CA was excellent for 5FC and VCZ but not for ICZ and FCZ.

Discussion

Epidemiological surveillance of the antifungal resistance of cryptococcal agents is relevant due to the potential burden, as well as the high lethality, presented by cryptococcosis in the Amazonian region. Cryptococcosis caused by *C. gattii* VGII is endemic in northern Brazil, and it is also the major type of meningitis seen in non-HIV infected patients in this region.[10,11] In the present study, 46% of the *C. gattii* VGII strains were from children and teenagers (5–15 years old) without detectable underlying risk factors for cryptococcosis. *C. neoformans* VNI is also endemic in this region and is mainly associated with meningitis in HIV-infected patients. However, this genotype may also infect individuals without predisposing factors.[10,11] In fact, 48% of the *C. neoformans* VNI strains analyzed were isolated from patients without HIV infection, including seven children/teenagers (3–17 years old, Supplementary Table 1).

All of the strains used in this study were preserved in culture collections for long periods of time. The preservation methods used could affect the phenotypic characteristics of these strains and therefore might affect the results of antifungal susceptibility

Table 2. Distribution of the MIC values for *Cryptococcus neoformans* VNI and *Cryptococcus gattii* VGII clinical strains and the essential agreement within 2 dilutions for the CLSI broth microdilution and Etest methods.

			N° of isolates for which the MIC (μg/mL)																											
Species (n° of isolates)	Antifungal agent	Methods	0.012	0.015	0.016	0.023	0.03	0.047	0.06	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32	48	EA%	*p*-values
C. neoformans VNI ($n = 62$)	AMB	CLSI													9		51		2											2.963×10^{-12}
		Etest	1			2	2	5	15	17	17	2	1																	
	5FC	CLSI															4		28		28		2						96.77	1
		Etest									1				1		3	1	3	12	19	14	6	1			1			
	ICZ	CLSI							9		47		5		1														93.55	0.203
		Etest					2	6	9	7	11	7	7	1	2	5	5													
	FCZ	CLSI																	16		42		4						96.77	0.346
		Etest																	3	8	13	12	17	4	3	1	1			
	VCZ	CLSI		1			22		35		2		2																100	1
		Etest			3	8	12	12	19	4	3		1																	
C. gattii VGII ($n = 37$)	AMB	CLSI													1		33		3										2.7	3.121×10^{-12}
		Etest				1		2	9	14	8	2		1																
	5FC	CLSI													1		4		11		16		4		1				72.97	0.0042
		Etest									1		1		7	6	4	7	6	1	3	1								
	ICZ	CLSI									10		20		7														94.87	1
		Etest						1	2	3	3	4	2	1	8	4	7	2												
	FCZ	CLSI																	2		15		11		8		1		94.87	1
		Etest																	1	2	4	7	4	2	7	6	2	2		
	VCZ	CLSI					1		5		7		23		1														94.87	1
		Etest				1	2	5	8	5	4	6	1	3	2															

AMB, amphotericin B; EA %, percentage essential agreement; FCZ, fluconazole; 5FC, flucytosine; ICZ, itraconazole; MIC, -minimum inhibitory concentration; VCZ, voriconazole.

Table 3. Distributions of the minimum inhibitory concentration values for *Cryptococcus neoformans* VNI and *Cryptococcus gattii* VGII clinical strains and percentage essential agreement within 2 dilutions for the CLSI- BMD and Vitek 2 methods.

			N° of isolates for which the MIC (μg/ml)															
Species (n° of isolates)	Antifungal agent	Methods	0.015	0.03	0.06	≤0.125	0.125	0.25	0.5	≤ 1	1	2	4	8	16	32	EA %	*P*-values
C. neoformans VNI ($n^{\circ} = 62$)	AMB	CLSI							9		51	2					100	1
		Vitek 2									61	1						
	5FC	CLSI									4	28	28	2			22.58	NA
		Vitek 2								48		14						
	FCZ	CLSI										16	42	4			61.29	NA
		Vitek 2								26		38						
	VCZ	CLSI	1	22	35		2	2									ND	NA
		Vitek 2				62												
C. gattii VGII ($n^{\circ} = 37$)	AMB	CLSI							1		33	3					100	1
		Vitek 2							4		29	3	1					
	5FC	CLSI							1		4	11	16	4	1		10.81	NA
		Vitek 2								33		4						
	FCZ	CLSI										2	15	11	8	1	89.19	1
		Vitek 2								4		15	7	8	2	1		
	VCZ	CLSI		1	7		7	23	1								2.7	NA
		Vitek 2				36		1										

ICZ is not included in the AST-YS01 card. AMB, amphotericin B; EA %, percentage essential agreement; FCZ, fluconazole; 5FC, flucytosine; MIC, minimum inhibitory concentration; NA, not applied; ND, not determined; VCZ, voriconazole.

testing. For this reason, we have tested some important phenotypic characteristics related to the virulence of *Cryptococcus* spp. to check whether these strains presented altered phenotypes. Our results demonstrated that even after 5– 11 years of preservation, these strains were able to maintain their virulence-related phenotypic traits.

When the CLSI-BMD or the Vitek 2 system was used for MIC determination, more than 97% of the *C. neoformans* VNI and *C. gattii* VGII strains showed WT phenotype to the azole antifungal drugs tested, indicating that strains within the ECVs of azoles are predominant in the Brazilian Amazon region. However, when analysed with the Etest, the same strains showed more n-WT phenotypes. One explanation for this difference is that the Etest method uses a more concentrated fungal inoculum, which can facilitate the detection of more elevated MICs for azole drugs. Because quorum-sensing molecules produced by *C. neoformans* cells, including pantothenic acid, can modulate the growth of this fungus,[34] a system with a higher cell density should have a higher concentration of these molecules, favoring the growth of *C. neoformans* even in the presence of fungistatic antifungal drugs. Most Brazilian studies with FCZ using the CLSI-BMD method give MIC values representing a WT phenotype, which is comparable to our study.[35–39] However, three of these previous studies have also revealed n-WT FCZ and ICZ strains.[35,37,38] Additionally, a study carried out in the city of Manaus in the state of Amazonas using the Etest method, revealed one environmental *C. neoformans* VNI isolate with a high MIC (48 μg/ml) for FCZ.[40] Another study using the Etest method and ICZ in the same region[40] showed that half of the environmental *C. neoformans* VNI strains tested were classified as n-WT.

The results of antifungal susceptibility testing of VGII to FCZ, as depicted by CLSI-BMD, are variable depending on the geographic origin of the strains. While studies with strains from northeastern Brazil present a few strains with high MICs,[36,37] MICs reported in studies from the midwestern and southern regions of Brazil are low.[38,41] Brazil is a large country, and molecular differences in *C. gattii* VGII isolates from different regions have been observed,[10] but whether a regional pattern of *C. neoformans* and *C. gattii* FCZ susceptibility exists still needs to be investigated. There have been no studies conducted in Brazil that used the Etest to determine the susceptibility of properly characterized *C. gattii* VGII strains to ICZ or VCZ. Favalessa et al.,[42] which examined nontyped *C. gattii* strains, presented very low MIC values for both drugs. Because *C. gattii* VGII predominates in the region studied by these authors, it is likely that at least

Table 4. Categorization of the strains according to the epidemiological cutoff values for *Cryptococcus neoformans* VNI and *Cryptococcus gattii* VGII and the corresponding categorical agreements of commercial methods with CLSI-BMD as the reference method.

		Antifungal agents: % of MIC values equal or greater than ECVs categorized as WT or n-WT and the CLSI-BMD reference method											
		AMB			5FC			ICZ			FCZ			VCZ		
Species (n° of isolates)	Methods	WT	n-WT	CA	WT	n-WT	CA	WT	n-WT	CA	WT	n-WT	CA	WT	n-WT	CA
C. neoformans VNI	CLSI	14.52	85.48	NA	100	–	NA	98.39	1.16	NA	100	–	NA	100	–	NA
($n° = 62$)	Etest	100	0	14.52	96.77	3.23	96.77	79.03	20.97	79.03	85.48	14.52	85.48	98.39	1.61	98.39
	Vitek 2	0	100	85.48	100	–	100	ND	ND	ND	100	–	100	100	–	100
C. gattii VGII	CLSI	91.89	8.11	NA	100	–	NA	100	–	NA	100	–	NA	97.29	2.71	NA
($n° = 37$)	Etest	100	0	91.89	100	–	100	64.86	35.14	64.86	94.59	5.41	94.59	86.49	13.51	86.49
	Vitek 2	89.19	10.81	89.19	100	–	100	ND	ND	ND	100	–	100	100	–	97.29

AMB, amphotericin B; CA, categorical agreement; ECVs, epidemiological cut-off values; FCZ, fluconazole; 5FC, flucytosine; MIC, minimum inhibitory concentration; NA, not applied; ND, not determined; n°, number of isolates; n-WT, non-wild type; VCZ, voriconazole; WT, wild type.

some of their *C. gattii* strains were VGII. A single study using EUCAST-BMD to determine the FCZ susceptibility of *C. gattii* VGII strains revealed that 25% of the included strains from the southern and mid-western regions of Brazil presented elevated FCZ MIC values.[43] It is important to note that the inoculum size used in the EUCAST-BMD method was higher than that used in the CLSI-BMD method.

Regarding AMB, the results obtained from the Etest compared with the CLSI-BMD or Vitek 2 were highly discordant as well. CLSI-BMD classified most of the VNI and VGII isolates as non-WT, and Vitek 2 classified all of the isolates as n-WT. The Etest presented completely discordant results and classified all strains tested as WT. Three studies on AMB susceptibility in this region using disk diffusion assays,[10] Etests,[41] and CLSI-BMD[39] revealed low MIC values in clinical and environmental *C. neoformans* VNI strains. Studies conducted in other Brazilian regions that used the CLSI-BMD to test AMB susceptibility reported values up to 2 μg/ml,[36–38] suggesting the occurrence of n-WT strains similar to what was observed in this study. Therefore, the discordances between AMB MIC values obtained by the different methods makes it difficult to determine the correct categorization of *C. neoformans* VNI. Studies conducted in other Brazilian regions did not report n-WT AMB susceptibility of *C. gattii* VGII strains.[36,38,40,41,43] On the other hand, a single study in which the majority of clinical *C. gattii* VGII strains tested were from the northeast region of Brazil reported AMB MICs of 2 μg /ml.[37] Considering that the northern and northeast regions of Brazil are endemic for *C. gattii* VGII cryptococcosis, further studies are necessary to monitor AMB susceptibility using the Vitek 2 system and/or the CLSI-BMD assay, as the Etest was not able to detect AMB n-WT strains.

The AMB inconsistencies demonstrated in this work were confirmed by the very low essential agreement (EA) observed for *C. neoformans* and *C. gattii* susceptibilities tested by Etest and CLSI-BMD. The AMB MIC range as determined by Etest depicted in this study is in accordance with several studies;[44–48] however, the AMB MIC range described here does not agree with those reported by some international studies[49,50] that described low MIC values for this antifungal drug. On the other hand, the studies of Aller et al.[44] and Dias et al.[47] present AMB MICs closer to the present work, as they also reported elevated MICs for AMB when the CLSI-BMD was employed for susceptibility testing. Taken together, these results suggest an inefficiency in the ability of the Etest to detect high AMB MICs, which leads to low EA values when n-WT strains are analyzed.

To the best of our knowledge, this is the first Brazilian study to compare CLSI-BMD and the Vitek 2 system to determine *Cryptococcus* spp. AMB MICs, and significant differences between the two methods were not observed. Our study agrees with the results of Mahabeer et al.[50] and, to a lesser extent, with the works of Ceijudo et al.[51] and Tewari et al.[49]

In this study, 5FC was highly active against all *C. neoformans* VNI strains as demonstrated by CLSI-BMD and Vitek 2. However, using the Etest, one strain was unaffected by this drug. Other Brazilian studies using the CLSI-BMD have also revealed strains that were not inhibited by high concentrations of 5FC.[36–38] MIC values of *C. gattii* VGII for 5FC are indicative of a WT phenotype, irrespective of the method. Similar results were observed in other studies,[36,38,41] with the exception of an investigation conducted with strains maintained in a fungal culture collection that reported MIC values > 64 μg/ml for *C. gattii* VGII using the CLSI-BMD method.[37]

In the present study, the low EA observed between CLSI-BMD and Vitek 2 for 5FC and *C. neoformans* VNI was based on the results of 14 strains whose MIC values were accurately reported by the Vitek 2 system. The other 48 strains were not included because they showed an MIC of < 1 μg/ml. This means that the exact MIC may assume any value lower than 1 μg/ml (i.e., 0.5 μg/ml, 0.25 μg/ml, 0.125 μg/ml, etc.) making the calculation of the EA imprecise. If we had assumed the value of 1 μg/ml for these strains, an EA similar to other studies would have resulted.[50,51] Therefore, we assume that when one method does not permit the determination of an exact MIC value within the same range of concentrations, as occurs with the Vitek 2 system, the EA calculation may suffer interference from these reports, and thus will not express the actual concordance between the methodologies. Regarding *C. gattii* VGII, this is the first study that compared these two methodologies for 5FC MIC determination. The same applies for the comparison between the Etest and CLSI-BMD methods for both *C. neoformans* VNI and *C. gattii* VGII.

Considering the MIC values determined by the CLSI-BMD method and the proposed *Cryptococcus* spp. ECVs,[32,33] more than 97% of the studied strains, which were collected in the Amazon region from 1995 to 2006, were categorized as WT for the azoles tested. A study of Latin American isolates from 2001 to 2008 showed a similar profile,[52] as well as a study of *C. gattii* VGII isolates from the Pacific Northwest outbreak in the United States.[53] The high frequency of *C. neoformans* VNI n-WT AMB isolates in the population described herein is noteworthy. This is relevant due to the high number of strains studied and their geographic origin in a large tropical region of the Amazon. There is a paucity of studies focusing on Amazon strains to which these results can be compared over time. Moreover, most of the Brazilian strains included in the *Cryptococcus* spp. ECV definition studies[32,33] were not from the northern region of the country. More studies are necessary to verify whether the Amazonian *C. neoformans* VNI strains present a different background of susceptibility to AMB.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

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

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