Original Article

Molecular typing and *in vitro* antifungal susceptibility of *Cryptococcus* spp from patients in Midwest Brazil

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Abstract

Introduction: Cryptococcosis is a systemic fungal infection that affects humans and animals, mainly due to *Cryptococcus neoformans* and *Cryptococcus gattii*. Following the epidemic of acquired immunodeficiency syndrome (AIDS), fungal infections by *C. neoformans* have become more common among immunocompromised patients. *Cryptococcus gattii* has primarily been isolated as a primary pathogen in healthy hosts and occurs endemically in northern and northeastern Brazil. We to perform genotypic characterization and determine the *in vitro* susceptibility profile to antifungal drugs of the *Cryptococcus* species complex isolated from HIV-positive and HIV-negative patients attended at university hospitals in Cuiabá, MT, in the Midwestern region of Brazil.

Methodology: Micromorphological features, chemotyping with canavanine-glycine-bromothymol blue (CGB) agar and genotyping by *URA5*-RFLP were used to identify the species. The antifungal drugs tested were amphotericin B, fluconazole, flucytosine, itraconazole and voriconazole. Minimum inhibitory concentrations (MICs) were determined according to the CLSI methodology M27-A3.

Results: Analysis of samples yelded *C. neoformans* AFLP1/VNI (17/27, 63.0%) and *C. gattii* AFLP6/VGII (10/27, 37.0%). The MICs ranges for the antifungal drugs were: amphotericin B (0.5-1 mg/L), fluconazole (1-16 mg/L), flucytosine (1-16 mg/L), itraconazole (0.25-0.12 mg/L) and voriconazole (0.06-0.5 mg/L). Isolates of *C. neoformans* AFLP1/VNI were predominant in patients with HIV/AIDS, and *C. gattii* VGII in HIV-negative patients. The genotypes identified were susceptible to the antifungal drugs tested.

Conclusion: It is worth emphasizing that AFLP6/VGII is a predominant genotype affecting HIV-negative individuals in Cuiabá. These findings serve as a guide concerning the molecular epidemiology of *C. neoformans* and *C. gattii* in the State of Mato Grosso.

Key words: Cryptococosis; *Cryptococcus* complex; antifungal drugs.

Introduction

Cryptococcosis is a severe systemic fungal infection affecting humans and a variety of animals. It is mainly caused by two species of yeasts of the genus *Cryptococcus*: *Cryptococcus neoformans* (serotypes A, D and AD) and *Cryptococcus gattii* (serotype B and C) [1]. Regarding molecular types, *C. neoformans* has been grouped into AFLP1/VNI and AFLP1A/VNII (serotype A), AFLP3/VNIII (serotype AD) and AFLP2/VNIV (serotype D), whereas *C. gattii* has been grouped into types AFLP1/VGI, AFLP6/VGII, AFLP5/VGIII and AFLP7/AFLP10/VGIV (serotypes B and C) [2]. Immunocompromised patients are more frequently affected by *C. neoformans* serotypes A and D. [3-5]

This species is distributed worldwide and is considered an important cause of morbidity and mortality in immunocompromised individuals and particularly among HIV-infected patients [3-5]. *C. neoformans* is associated with organic matter in the habitats of pigeons, birds in captivity and domestic environments, in domestic dust and decomposing wood and in different species of hollow trees [6-8].

Until recently, it was believed that the species *C. gattii* was confined to regions with tropical and subtropical climates, like Australia and New Zealand, where it is associated with species of *Eucalyptus* spp.

However, it was described in a recent outbreak on Vancouver Island, Canada, a temperate region, suggesting that the species *C. gattii* has adapted to different environmental conditions [9]. It has also been isolated from clinical specimens in several other areas of the world, including Mexico, parts of Latin America, Europe, Southern California, Hawaii, India and Africa [10-15].

In Brazil, cryptococcosis caused by *C. neoformans* occurs in all regions of the country [5,16-20]. However, *C. gattii* has emerged as a primary pathogen infecting immunocompetent individuals, particularly children, adolescents and young adults in northeastern Brazil, where cryptococcosis is characterized by high mortality rates [18,20-22].

Infection is caused by the inhalation of viable propagules of these yeasts directly from the environment. After invading the lung tissue, hematogenous dissemination occurs with a predisposition for the central nervous system [23]. Meningitis and meningoencephalitis are the most common clinical manifestations in cases of cryptococcal meningitis caused by *Cryptococcus* spp, while *C. gattii* shows a propensity to cause cryptococcomas, focal CNS lesions and important neurological sequelae [14,15,23,24].

The purpose of this study was to characterize the *Cryptococcus* species complex and its molecular types derived from clinical isolates obtained from patients diagnosed with cryptococcosis admitted to the university hospitals of Cuiabá, MT, using chemotyping in canavanine-glycine-bromothymol blue medium, genotypic characterization by PCR and *URA5*-RFLP. The *in vitro* susceptibility profiles of *C. neoformans* and *C. gattii* to amphotericin B, fluconazole, flucytosine, itraconazole and voriconazole. were also determined.

Methodology

Cryptococcus identification

Between August 2010 and July 2013, clinical isolates of *Cryptococcus* spp from patients admitted to the university hospitals of Cuiabá (General University Hospital, HGU; Julio Muller University Hospital, HUJM), were identified in the Mycology Research Laboratory of the Federal University of Mato Grosso (UFMT). Identification was achieved by: visualizing micromorphological features in direct examination with India ink; biochemical tests, phenol oxidase positive on Niger seed agar and urease positive; and phenotypic characterization by chemotyping on canavanine-glycine-bromothymol blue (CGB) medium.

Genotypic characterization

Genotypic identification was performed by polymerase chain reaction (PCR) using paired primers CNB49A (5'ATTGCGTCCATCCAACCGTTATC-3 ') and CNB49S (5'ATTGCGTCCAAGTGTTGTTG-3'), specific for *C. gattii* and CNA70S: (5 'ATT GCG GAG CTC TCCACCAAG 3') and CNA70A (5 'ATT GCG TCC ATG TTA CGTGGC 3') specific for *C. neoformans* [25,26].

DNA extraction

For DNA extraction, the protocol described by Del Poeta *et al* (1999) [27] was used with modifications. Lysis buffer (0.5 mM EDTA pH 8.0, 100 mM NaCl, 10 mM Tris pH 8.00, and 0.5% SDS) was used, with 0.05 g of glass beads. The tubes were initially agitated in a vortex for 5 minutes, then boiled at 100°C for 5 minutes and finally, centrifuged at 16,000 *g* for 5 minutes. The aqueous phase was extracted with 0.5 volume of buffered phenol and chloroform, shaken gently for 5 minutes. This was then centrifuged (6,000 *g* for 10 minutes) and the nucleic acid content present in the upper phase was collected and precipitated in the presence of 0.2 M NaCl, pH 5.2, and 1 mL isopropanol for 16 hours at -20°C, and then centrifuged (10,000 *g* for 10 minutes), washed with1 mL of 70% ethanol and dried. Incubation was performed overnight at -20°C to achieve precipitation. The DNA was collected by centrifugation at 16,000 *g* for 5 minutes, after which the supernatant was discarded. The pellet was washed with 1 mL of cold 70% ethanol and resuspended in 0,05 mL of MilliQ water. Subsequently, the DNA was treated with RNAse A for 1 hour at 37°C. The quality and integrity of the DNA was analyzed by electrophoresis in 1.0% agarose gel at 100 V per cm with the aid of a transilluminator (Loccus, São Paulo, Brazil).

Molecular typing

Twenty-seven Brazilian clinical isolates were typed by *URA5*-RFLP and PCR-fingerprinting using the minisatellite-specific core sequence of the wild-type phage M13. PCR of the gene *URA5* was performed in a final volume of 50 μL. Each reaction contained 50 ng of DNA, 1x PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH8.8 at 25°C), 0.1% Tween-20 – (Bioline Inc., Taunton, USA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM

magnesium chloride, 1.5 U BioTaq DNA polymerase (Bioline Inc., Taunton, USA), and 50 ng of each *URA5* primer (5' ATGTCCTCCCAAGCCCTCGACTCCG 3') and SJ01 (5' TTAAGACCTCTGAACACCGTACTC 3') [28]. PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) at 94°C with 2 minutes initial denaturation, 45 s denaturation at 94°C, 1 minute annealing at 61°C, 2 minutes extension at 72°C, and final extension cycle for 10 minutes at 72°C. A total of 30 μl of PCR products were double digested with *Sau*96I (10 U/μL) and *Hha*I (20 U/μL) for 3 hours, 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 provided by the Mycology laboratory of the Institute of Clinical Research Evandro Chagas – Oswaldo Cruz Foundation and included WM 148 (VNI), WM 626 (VNII), WM 628 (VNII), 629 (VNIV), WM 179 (VGI), WM 178 (VGII), WM 161 (VGIII) and WM 779 (VGIV).

Antifungal susceptibility testing

The *in vitro* susceptibility profiles of *C. neoformans* and *C. gattii* against antifungal agents were determined by using the reference method of broth microdilution, in accordance with document M27-A3 of the CLSI. Cutoff points for *C. neoformans* and *C. gattii* have not been established by the CLSI, so those described by CLSI document M27-A3 for *Candida* spp were used, as previously reported [30-

33]. The antifungal drugs tested were amphotericin B, fluconazole, flucytosine, itraconazole and voriconazole.

Results

Among the 27 patients diagnosed with cryptococcosis during the study period, 27 strains of *Cryptococcus* spp were isolated. Fourteen patients were HIV-positive, with 13 presenting isolates of *C. neoformans* VNI and one of *C. gattii* VGII. Thirteen patients showed HIV negative serology, of which 10 presented isolates of *C. gattii* and three of *C. neoformans*. Fifteen of the 27 infected individuals were male, with ages ranging from 6 to 73 years (mean age, 37.9 years).

Cryptococcus neoformans was the most prevalent isolate among HIV-infected individuals and *C. gattii* was the most prevalent among HIV-negative individuals. The antifungal drug susceptibility tests showed *in vitro* activity against isolates of *Cryptococcus* spp and the MICs ranges to *C. neoformans* were: amphotericin B 0.5 - 1 mg/L; fluconazole 1– 16 mg/L; flucytosine 0.5-8 mg/L; itraconazole 0.03 – 0.25 mg/L and voriconazole 0.06-0.5 mg/L MICs ranges against *C. gattii* were: amphotericin B 0.5 - 1 mg/L; fluconazole 1 – 16 mg/L; flucytosine 1 - 16 mg/L; itraconazole 0.03 – 0.5 mg/L and voriconazole 0.06-0.5 mg/L. The minimum inhibitory concentrations (MICs) and MIC_{50} and MIC_{90} values are presented in Table 1.

Table 1. *In vitro* susceptibility of the genotypes of *Cryptococcus* spp to fluconazole, itraconazole, voriconazole, flucytosine and amphotericin B in isolates from HIV-positive and HIV-negative patients.

Genotype and antifungal drug	MIC range	*MIC_{50}	*MIC_{90}	**GM
	(mg/L)	(mg/L)	(mg/L)	(mg/L)
C. neoformans VNI (n = 17)				
Amphotericin B	0.5-1	0.5	1	0.67
Fluconazole	1-16	4	8	4.34
Flucytosine	0.5-8	4	4	2.77
Itraconazole	0.03-0.25	0.12	0.25	0.09
Voriconazole	0.06-0.5	0.5	0.5	0.28
C. gattii VGII (n = 10)				
Amphotericin B	0.5-1	0.5	1	0.71
Fluconazole	1-16	8	16	7.46
Flucytosine	1-16	8	8	4.92
Itraconazole	0.03-0.5	0.25	0.5	0.22
Voriconazole	0.06-0.5	0.5	0.5	0.28

* MIC50 and MIC90, the concentration capable of inhibiting the growth of isolates by 50% and 90%, respectively. **GM: geometric means.

Discussion

Epidemiological studies addressing the molecular features of species belonging to the genus *Cryptococcus* have been conducted extensively in numerous parts of the world [2,34-36]. However, in Brazil, molecular epidemiology studies are still required to elucidate the distribution of molecular types in all five Brazilian regions. Despite this, the few epidemiological studies conducted in Brazil have demonstrated differences in the distribution of the genotypes of these species in these regions [17-21,36]. In Mato Grosso, this is the first report describing the molecular types circulating in the state capital of Cuiabá. This study demonstrated the prevalence of cryptococcal meningitis by *C. neoformans* VNI among HIV-infected patients and *C. gattii* VGII in patients presenting negative serology for HIV.

Cryptococcosis has become more frequently diagnosed among HIV/AIDS patients and is considered an important cause of morbidity and mortality. *C. neoformans* is the species with the largest number of isolates among species belonging to the *Cryptococcus* complex [5,16-18,37]. Currently, an increasing number of cases of cryptococcosis have been reported in patients without HIV. Regarding this finding, we recommend testing for *C. gattii* as a primary pathogen, since it affects apparently healthy individuals, especially in northern and northeastern Brazil [21,22,36].

In Brazil, *C. neoformans* predominates in clinical isolates from HIV/AIDS patients, particularly in the southern, southeastern and mid-western regions [9,16-18,20]. Of the *C. neoformans* species analyzed in this study, all showed the molecular type VNI, a prevalence observed by other researchers [8,10,19,20]. In the 1990s, during the outbreak that occurred on Vancouver Island and nearby areas in Canada and the USA, the genotype *C. gattii* VGIIa / VGIIc emerged as a primary pathogen, illustrating that the species is not only present in tropical and subtropical regions, but also in other geographic areas, including those with temperate climates. This outbreak indicated that exposure to environmental sources, such as trees and soil contaminated by this yeast, can lead to cryptococcosis infection in humans and animals [38,39,40]. In a study analyzing environmental samples in Cuiabá, MT, Anzai *et al*. [41] also isolated the genotype *C. gattii* VGII, which is similar to that detected in the clinical isolates analyzed in this work.

Santos *et al*. [21] studied 43 cases of meningitis caused by *Cryptococcus* spp, in which *C. gattii* was the most common isolate among HIV-negative patients (19/29; 65.5%). The genotype VGII (25/56, 44.6%) was the most frequently isolated, as reported previously [36-42], and in agreement with this work. Different results have been observed in southern Brazil, with Casali *et al*. [18] reporting 11 isolates of *C. gattii* serotype B type VGIII among the clinical samples they studied. In Goiânia, MS, Souza et al. [20] identified *C. gattii* serotype B type VGIII in four clinical samples.

In vitro susceptibility tests are not routinely performed in Brazilian laboratories, particularly in the public health services, even though these tests are extremely useful for selecting the appropriate therapy. In this study, clinical isolates of *C. neoformans* and *C. gattii* were susceptible to the antifungal agents tested (Table 1).

Studies conducted in several parts of the world have shown low MIC_{50} and MIC_{90} values for fluconazole against *C. neoformans* VNI [43-45]. In this study, the MIC_{50} and MIC_{90} values for fluconazole were 4-8 mg/L; however, higher values (2-128 mg/L) have been reported for isolates of *C. neoformans* VNI [46].

High MIC values ($\geq$ 64 mg/L) have been reported for fluconazole against C. gattii [44,47], in contrast to this study, which determined values $\leq$ 16 mg/L. Hagen *et al*. [48] analyzed 350 isolates of *C. gattii* originating from clinical, environmental and animal sources and reported that MIC values for flucytosine and fluconazole were higher, i.e. these antifungal drugs showed less activity *in vitro* against *C. gattii* compared with isavuconazole, itraconazole, posaconazole, and voriconazole. Regarding new azoles, voriconazole demonstrated strong activity against the genotypes, even against isolates that presented diminished susceptibility to fluconazole. In this study, voriconazole also showed low MIC values against *C. gattii* and *C. neoformans*, suggesting that voriconazole could be an important drug for the treatment of cryptococcosis in cases of resistance to fluconazole, and due to the toxic effects related to amphotericin B. Despite the promising *in vitro* results obtained for voriconazole, numerous other studies are required to determine the correlation between *in vitro* susceptibility and therapeutic success *in vivo*.

Following their analysis of susceptibility profiles of *C. gattii* genotypes, Chong *et al*. [49] reported higher MIC values for fluconazole ($\geq$ 64 µg/mL) against *C. gattii* VGII than against *C. gattii* VGIII and VGI [48]. Iqbal *et al*. [50] reported differences in the susceptibility profiles of the molecular types of *C. gattii*, having determined higher MIC values for the

VGII subtypes than the VGI and VGIII subtypes. In Brazil, Souza et al. [20] reported low MIC values for voriconazole and amphotericin B against isolates of *C. gattii*. It is worth highlighting that *C. gattii* VGIII was identified in this study and that the MIC values obtained for amphotericin B were low, in agreement with those in the literature [47,50,51], considering the cutoffs used by Nguyen and Yu [30] and Lozano-Chui *et al.* [31]. For voriconazole, MIC values against *C. gattii* (range = 0.06 – 0.5 mg/L were in agreement with those presented by Espinel-Ingroff *et al* [53], whereas against *C. neoformans* MIC values (range = 0.06–0.5 mg/L), were lower compared with those of the same study.

The majority of studies addressing *in vitro* susceptibility testing for amphotericin B against *C. neoformans* and *C. gattii* have shown that many of these isolates are susceptible at MIC values ≤ 1 mg/L [47,48,50-53], similar to the MIC values obtained in this work. However, the literature reports MIC values for amphotericin B ≥ 1 µg/mL, such as Lozano-Chui *et al.* [31], who reported MIC values of 3-4 mg/L, which were associated with treatment failure.

Given the severity of infection by *Cryptococcus gattii* and the potential neurological sequelae that affect not only immunocompromised, but also healthy individuals, the adequation of laboratory facilities to provide early diagnosis of cryptococcosis in Brazil is essential. Moreover, the importance of performing molecular typing of the species of *C. gattii* should be emphasized, given literature findings indicating the existence of differences in the *in vitro* susceptibility profiles. Understanding the molecular types circulating in different Brazilian regions and their correlation with the minimal inhibitory concentrations values obtained for the antifungals most frequently used in medical practice should elucidate aspects of this important enigmatic puzzle, the Cryptococcus complex.

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