Mycopathologia (2013) 176:267–272
DOI 10.1007/s11046-013-9676-6

First Molecular Typing of Cryptococcemia-Causing *Cryptococcus* in Central-West Brazil

Rosianne Assis de Sousa Tsujisaki · Anamaria Mello Miranda Paniago · Manoel Sebastião da Costa Lima Júnior · Débora de Souza Olartechea de Alencar · Fernanda Luíza Espinosa Spositto · Maína de Oliveira Nunes · Luciana Trilles · Marilene Rodrigues Chang

Received: 25 March 2013 / Accepted: 21 June 2013 / Published online: 12 July 2013

Abstract Molecular epidemiology studies on cryptococcemia are limited. This study aimed to describe the clinical features of patients with bloodstream infections by *Cryptococcus* sp. in a public tertiary hospital in Mato Grosso do Sul, as well as identify the fungus' molecular type and determine its antifungal susceptibility. Molecular typing was performed using *URA5* restriction fragment length polymorphism PCR, and antifungal susceptibility was determined by microdilution method standardized by the Clinical and Laboratory Standards Institute. Over 14 years, 48 patients were diagnosed with cryptococcemia. The majority (72.9 %) was male with a median age of 40 years; 81.3 % of the patients had HIV/AIDS and 72.9 % died. *Cryptococcus neoformans* was the most commonly isolated species (97.9 %). Molecular analysis identified the genotypes *C. neoformans* VNI (93.7 %), *C. neoformans* VNII (4.2 %), and *Cryptococcus gattii* VGII (2.1 %). In vitro, these fungi were not resistant to fluconazole, itraconazole, voriconazole, and amphotericin B. This is the first description of the molecular types of cryptococcemia agents in central-west Brazil. Its high lethality, especially in HIV-negative patients, suggests that early diagnosis and prompt antifungal therapy are crucial for a good clinical outcome.

R. A. de Sousa Tsujisaki · D. de Souza Olartechea de Alencar · F. L. E. Sposito · M. R. Chang (✉)
Microbiological Research Laboratory, Biology and Health Sciences Center, Federal University of Mato Grosso do Sul, Cidade Universitária, Campo Grande, Mato Grosso do Sul CEP 79070-900, Brazil
e-mail: marirchang@yahoo.com.br

A. M. M. Paniago
Medical School, Federal University of Mato Grosso do Sul, Cidade Universitária, Caixa Postal 549, Unidade 9, Campo Grande, Mato Grosso do Sul CEP 79070-900, Brazil

M. S. da Costa Lima Júnior
Federal Institute of Mato Grosso do Sul, 473, km 23, Fazenda Sta. Bárbara, s/n. Caixa Postal 144, Campus Nova Andradina, Nova Andradina, Mato Grosso do Sul CEP 79750-000, Brazil

M. de Oliveira Nunes
Mycology Laboratory, University Hospital Group, Hospital Universitário Maria Aparecida Pedrossian, Av. Felinto Muller, 355, Campo Grande, Mato Grosso do Sul CEP 79080-190, Brazil

L. Trilles
Laboratory of Mycology, Institute of Clinical Research Evandro Chagas, Fiocruz, Av. Brasil, 4365, Manguinhos, Rio de Janeiro, RJ CEP 21040-900, Brazil

Keywords Cryptococcemia · *Cryptococcus neoformans* · *Cryptococcus gattii* · Molecular typing · HIV-negative

Introduction

Cryptococcosis is a systemic fungal disease associated with high lethality. It occurs in immunocompetent individuals, but more frequently in immunocompromised patients mainly with HIV/AIDS [1]. Cryptococcal infections are primarily acquired by inhaling propagules dispersed in the air from dried bird droppings and decomposing wood. The primary lung infection can spread to other sites through hematogenic dispersion, with specific tropism for the central nervous system [1]. The etiological agents that cause cryptococcosis are distributed worldwide and belong to the *Cryptococcus neoformans* complex, which comprises two species: *C. neoformans* (serotypes A, D, and AD; molecular types VNI, VNII, VNIII, and VNIV) and *Cryptococcus gattii* (serotypes B and C; molecular types VGI, VGII, VGIII, and VGIV) [2, 3].

Few studies in the literature have specifically addressed bloodstream infections by *Cryptococcus* [4, 5] and sporadic cases are reported [6–9]. Besides, the presence of cryptococcemia is often associated with a poor prognosis with a high risk of death [4].

In the last years, azole (mostly fluconazole) antifungal susceptibility and molecular types differences have been reported for *C. neoformans* and *C. gattii* [10, 11]. Thus, this study aimed to describe clinical features from patients with cryptococcemia, identify the molecular type, and determine the in vitro antifungal susceptibility of the etiological agents.

Materials and Methods

Study Design

The study was performed with patients with cryptococcemia diagnosed at a public reference hospital in Campo Grande, Mato Grosso do Sul, Brazil, between July 1998 and July 2012. *Cryptococcus* sp. was isolated from the blood culture and identified in the Mycology Laboratory by the observation of dark brown yeast colonies on niger seed agar. The species were identified in canavanine–glycine–bromothymol blue medium [12] and molecularly by *URA5* restriction fragment length polymorphism PCR (*URA5*-RFLP). Cryptococcemia was considered when *Cryptococcus* sp. was isolated from blood cultures independent of the isolation from other sites.

Demographic, clinical, and laboratory data were obtained from medical records, including sex, age, underlying diseases, clinical manifestations, $CD4^+$ cell counts, and clinical outcome.

Molecular Typing

Molecular typing was performed using *URA5*-RFLP according to Meyer et al. [3]. The following reference strains were used for comparisons: *C. neoformans* WM 148 (serotype A, VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), and WM 629 (serotype D, VNIV) as well as *C. gattii* WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 175 (serotype B, VGIII), and WM 779 (serotype C, VGIV) [3, 13].

DNA was extracted based on a protocol from the Latin American Cryptococcal Network Project modified from Ferrer et al. [14]. The *URA5* gene was amplified by PCR using the primers URA5 (5′ ATGTCCTCCCAAGCCCTCGACTCCG 3′) and SJ01 (5′ TTAAGACCTCTGAACACCGTACTC 3′) (IDT, USA). The amplified PCR product was double digested using *Hha*I (20 U/μL) (New England Biolabs, USA) and *Sau*96I (5 U/μL) (New England Biolabs, USA) enzymes. The RFLP profiles were analyzed visually by comparison with the reference strains [3].

Antifungal Susceptibility Testing

Antifungal susceptibility was determined by broth microdilution method according to the Clinical and Laboratory Standards Institute—CLSI M27-A3 document [15]. The antifungals fluconazole (Sigma, USA) (0.125–64 μg/mL), itraconazole (Sigma, USA), voriconazole (Pfizer, USA), and amphotericin B (Sigma, USA) (0.015–8 μg/mL) were tested. The tests were performed in duplicate. Strains of *Candida*

parapsilosis ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls.

For the azoles, the minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that reduced fungal growth by 50 %. For amphotericin B, it was defined as the lowest concentration that inhibited fungal growth by 100 %.

Because the CLSI did not define endpoints for *Cryptococcus* sp., the endpoints previously used by other investigators were used in this study. The following values were considered resistant: fluconazole MIC $\geq$16 μg/mL [16]; itraconazole and voriconazole MIC $\geq$1 μg/mL [17]; and amphotericin B MIC $\geq$2 μg/mL [18].

Data Analysis

Demographic, clinical, and laboratory data were analyzed. Percentage and median were determined using the Epi Info™ 3.5.1 program [19], and the geometric mean of MICs for each antifungal was calculated using BioEstat® 5.3 software [20].

Ethical Aspects

This study was approved by the Research Ethics Committee of the Federal University of Mato Grosso do Sul.

Results

During the study period (1998–2012), 48 patients were diagnosed with cryptococcemia. Thirty-five (72.9 %) patients were male, and the ages ranged from 10 to 90, with a median of 40 years. The most common age (31.3 %) range was 35–49 years. The majority (62.5 %) of patients were from Campo Grande-MS, and 18 (37.5 %) were from other cities of Mato Grosso do Sul, most of which (87.5 %) living in urban areas.

The underlying diseases most commonly observed were HIV/AIDS (39; 81.3 %), diabetes mellitus (6; 12.5 %), and hepatic cirrhosis (2; 4.2 %). Among the HIV-positive patients, 24/25 (96.0 %) had CD4 levels lower than 100 cells/mm^3. In five patients (10.4 %), HIV serology was unknown. Overall clinical findings are summarized in Table 1.

Cryptococcus was also isolated from other clinical samples, including 24 (50.0 %) cerebrospinal fluid, 8 (16.7 %) urine, two (4.2 %) bronchial lavage, one (2.1 %) sputum, oropharyngeal fluid, tracheal aspirate, and bone marrow aspirate. The most common clinical manifestations were anemia (91.7 %), fever (83.3 %), weight loss (58.3 %), vomiting (58.3 %), cough (58.3 %), and headache (56.3 %). The hospital stay ranged from 1 to 82 days with a median of 16 days. Twenty-one (43.8 %) patients were admitted to the adult intensive care unit (ICU). Twenty-six out of the 39 HIV-positive patients and all four HIV-negative patients died. The etiological agent was *C. neoformans* in 47 (97.9 %) patients. In molecular typing were identified 45 *C. neoformans* VNI, two VNII, and one *C. gattii* VGII. All 48 strains of *C. neoformans* and *C. gattii* were sensitive to

Table 1 Clinical features of 48 cases of cryptococcemia [University Hospital, UFMS, Campo Grande, MS, Brazil (July 1998–2012)]

Clinical features	*n*	%
Clinical manifestations		
General manifestations		
Anemia	44	91.7
Fever	40	83.3
Weight loss	28	58.3
Diarrhea	20	41.7
Abdominal pain	19	39.6
Sepsis	15	31.3
Neurological manifestations		
Vomiting	28	58.3
Headache	27	56.3
Changes in consciousness	21	43.8
Nausea	20	41.7
Changes in vision	15	31.3
Stiff neck	12	25.0
Respiratory manifestations		
Cough	28	58.3
Dyspnea	23	47.9
Clinical conditions (or underlying diseases)		
HIV/AIDS	39	81.3
Diabetes mellitus	6	12.5
Cancer	2	4.2
Corticoid previous use	2	4.2
Cirrhosis	2	4.2
No reported	2	4.2

The patients had one or more clinical features

Table 2 Minimum inhibitory concentrations (MIC) of 48 *Cryptococcus* sp. strains against the antifungals tested

Antifungals	MIC (μg/mL)			
	Range	50 %	90 %	Geometric mean
Fluconazole	0.25–4.0	2.0	2.0	1.4768
Itraconazole	0.015–0.5	0.06	0.125	0.0653
Voriconazole	0.015–0.06	0.03	0.06	0.0279
Amphotericin B	0.125–1.0	0.25	0.5	0.3536

fluconazole, itraconazole, voriconazole, and amphotericin B. The minimum inhibitory concentrations, MIC50 % and MIC90 %, as well as the geometric means are shown in Table 2.

Discussion

Cryptococcosis is recognized as an important fungal disease globally [21]. *Cryptococcus* sp. isolation from the bloodstream is not rare, but few clinical/epidemiological studies have examined its role and the underlying etiological agents. Previous studies reported blood culture as an important diagnostic method for cryptococcosis in AIDS patients [22, 23].

In this study, *Cryptococcus* sp. was mainly isolated from the bloodstream of males (72.9 %), which corroborated the studies by Jean et al. [4] and Monteiro et al. [22], who described 78.8 and 81.8 % prevalences in males, respectively. Similar to previous reports on cryptococcosis, the majority of patients were adults [23–26]. Over the 14 years of this study, only one child who was a HIV virus carrier had cryptococcemia diagnosis, which suggests that such a clinical presentation is rare in the hospital studied. This result is consistent with Pasqualotto et al. [5], who described a bloodstream infection in only one immunocompromised child out of the 28 cryptococcemia cases. In Brazil, most of the cryptococcosis cases in children have been recorded in the north and northeast regions [27, 28]. According to Corrêa et al. [27], this observation may be related to the endemic nature of *C. gattii*-mediated cryptococcosis in the region. All patients with cryptococcemia were from Mato Grosso do Sul; thus, cryptococcosis can be considered autochthonous mycosis in this state.

It is well-established in the literature that HIV/AIDS is an important risk condition for cryptococcosis [23, 29, 30] and may explain the higher number of cryptococcemia cases among HIV carriers in this study. Similar to the observations by Pasqualotto et al. [5], those patients were highly immunocompromised because 96.0 % of them showed $CD4^+$ T lymphocyte counts at less than 100 cells/mm^3.

The clinical diagnosis of cryptococcemia is difficult by the fact the clinical signs vary greatly due to infection in other organs [23]. Previous studies have shown that patients with cryptococcemia have a high fever [6–9], which was also observed in our study. Thus, fever with tremors and chills in immunocompromised patients may be an alert for suspecting cryptococcemia.

Cryptococcus reaches many organs with a specific tropism for the central nervous system via hematogenic dissemination. According to Rozenbaum and Gonçalves [23], cryptococcemia typically precedes by invading the CNS and can persist for up to 16 weeks despite treatment. The present study isolated *Cryptococcus* from the spinal fluid in 50.0 % of patients with positive blood cultures. This result is consistent with authors who have described meningoencephalitis as the most frequent manifestation of cryptococcosis [24, 25, 29].

The severity of cryptococcemia and the consequent need for intensive care were evidenced by the fact that 43.8 % of patients were admitted to the ICU of the University Hospital. The higher lethality rate observed in the HIV-negative patients indicates that a *Cryptococcus* bloodstream infection is typically fatal, especially in elderly patients with diabetes mellitus, cancer, or corticoid use over an extended time. According to Jean et al. [4], *Cryptococcus* isolation from blood cultures is a condition with a high risk of death. Therefore, clinical suspicion and blood culture requests soon after admission are essential for prognosis.

Similar to previous studies, *C. neoformans* VNI was the most commonly isolated molecular type [3, 13, 25,

26] followed by *C. neoformans* VNII and *C. gattii* VGII. The VGII molecular type, which is common in immunocompetent individuals and in the north as well as northeast Brazil [13], was isolated from an aboriginal patient who also had diabetes mellitus. Freire et al. [25] isolated only *C. neoformans* from blood; three (5.3 %) were molecular type VNI, and one (1.8 %) was type VNII. *C. neoformans* VNII as cryptococcemia agent is very rare in the literature, as observed herein (two cases), besides this is the first description of the VGII molecular type in patients with cryptococcemia in central-west Brazil.

In our study, all *Cryptococcus* isolates were sensitive to the antifungals tested. Although rare, *Cryptococcus* resistance to azoles [31–33] and amphotericin B [18, 31, 33] has been reported. The results herein indicate that bloodstream *Cryptococcus* infection is fatal in most of the cases. Therefore, early diagnosis and prompt antifungal treatment are essential for improving the patients' outcome.

Acknowledgments The authors thank The HU/UFMS mycology laboratory team for providing *Cryptococcus* spp. strains. Financial support was from Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior e Pró-Reitoria de Pesquisa e Pós-Graduação da Universidade Federal de Mato Grosso do Sul.

References

1. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology; 1998.
2. Kwon-Chung KJ, Varma A. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? FEMS Yeast Res. 2006;6(4):574–87.
3. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E, IberoAmerican Cryptococcal Study Group. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003;9(2):189–95.
4. Jean SS, Fang CT, Shau WY, Chen YC, Chang SC, Hsueh PR, et al. Cryptococcaemia: clinical features and prognostic factors. QJM. 2002;95(8):511–8.
5. Pasqualotto AC, Severo CB, Oliveira FM, Severo LC. Cryptococcemia: an analysis of 28 cases with emphasis on the clinical outcome and its etiologic agent. Rev Iberoam Micol. 2004;21(3):143–6.
6. Choi HY, Choe JK, Lee YB. Hodgkin's disease complicated by cryptococcemia—one case report. Yonsei Med J. 1970; 11(2):173–81.
7. Fujita Y, Kasahara K, Uno K, Konishi M, Maeda K, Yoshimoto E, et al. Amphotericin B-induced nephrogenic diabetes insipidus in a case of cryptococcemia. Intern Med. 2005;44(5):458–61.
8. Yehia BR, Eberlein M, Sisson SD, Hager DN. Disseminated cryptococcosis with meningitis, peritonitis, and cryptococcemia in a HIV-negative patient with cirrhosis: a case report. Cases J. 2009; doi:10.1186/1757-1626-2-170.
9. Zaharatos GJ, Behr MA, Libman MD. Profound T-lymphocytopenia and cryptococcemia in a human immunodeficiency virus-seronegative patient with disseminated tuberculosis. Clin Infect Dis. 2001;33(11):e125–8.
10. Chong HS, Dagg R, Malik R, Chen S, Carter D. In vitro susceptibility of the yeast pathogen *Cryptococcus* to fluconazole and other azoles varies with molecular genotype. J Clin Microbiol. 2010; doi:10.1128/JCM.01271-10.
11. Trilles L, Meyer W, Wanke B, Guarro J, Lazéra M. Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans*/*C. gattii* species complex. Med Mycol. 2012; doi:10.3109/13693786.2011.602126.
12. Kwon-Chung KJ, Polacheck I, Bennett JE. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J Clin Microbiol. 1982;15(3):535–7.
13. Trilles L, Lazéra MS, Wanke B, Oliveira RV, Barbosa GG, Nishikawa MM, et al. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Mem Inst Oswaldo Cruz. 2008;103(5):455–62.
14. Ferrer C, Colom F, Frasés S, Mulet E, Abad JL, Alió JL. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J Clin Microbiol. 2001;39(8):2873–9.
15. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3. Wayne: Clinical and Laboratory Standards Institute; 2008.
16. Aller AI, Martin-Mazuelos E, Lozano F, Gomez-Mateos J, Steele-Moore L, Holloway WJ, et al. Correlation of fluconazole MICs with clinical outcome in cryptococcal infection. Antimicrob Agents Chemother. 2000;44(6):1544–8.
17. Souza LKH, Fernandes OFL, Kobayashi CCBA, Passos XS, Costa CR, Lemos JA, et al. Antifungal susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* in Goiânia city, Goiás, Brazil. Rev Inst Med Trop São Paulo. 2005;47(5):253–6.
18. Lozano-Chiu M, Paetznick VL, Ghannoum MA, Rex JH. Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: performances of three different media assessed by using E-test and National Committee for Clinical Laboratory Standards M27-A methodologies. J Clin Microbiol. 1998;36(10):2817–22.
19. Centers for Disease Control and Prevention. Epi Info™ versão 3.5.1. A Word processing, database and statistics program for public health. Atlanta: CDC; 2008. http://wwwn.cdc.gov/epiinfo/. Accessed 23 July 2012.
20. Ayres M, Ayres Júnior M, Ayres DL, Santos AAS. BioEstat® versão 5.3: aplicações estatísticas nas áreas das ciências biomédicas. Belém: ONG Mamirauá; 2007.
21. Del Poeta M, Chaturvedi V. *Cryptococcus* and cryptococcosis in the twenty-first century. Mycopathologia. 2012; doi:10.1007/s11046-012-9544-9.
22. Monteiro DU, Brum TF, Noal CB, Righi RA, Santos ER, Oliveira LTO, et al. Prevalência de Candida e Cryptococcus

em hemoculturas oriundas de pacientes do Hospital Universitário de Santa Maria, RS (HUSM) no ano de 2006. Saúde (Santa Maria). 2011;37(2):81–8.
23. Rozenbaum R, Gonçalves AJR. Clinical epidemiological study of 171 cases of cryptococcosis. Clin Infect Dis. 1994; 18(3):369–80.
24. Lindenberg ASC, Chang MR, Paniago AMM, Lazéra MS, Moncada PMF, Bonfim GF, et al. Clinical and epidemiological features of 123 cases of cryptococcosis in Mato Grosso do Sul, Brazil. Rev Inst Med Trop São Paulo. 2008;50(2):75–8.
25. Freire AKL, Bentes AS, Sampaio IL, Matsuura ABJ, Ogusku MM, Salem JI, et al. Molecular characterisation of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil. Mycoses. 2012; doi:10.1111/j.1439-0507.2012.02173.x.
26. Santos WRA, Meyer W, Wanke B, Costa SPSE, Trilles L, Nascimento JLM, et al. Primary endemic*Cryptococcosis gattii* by molecular type VGII in the state of Pará, Brazil. Mem Inst Oswaldo Cruz. 2008;103(8):813–8.
27. Corrêa MPSC, Oliveira EC, Duarte RRBS, Pardal PPO, Oliveira FM, Severo LC. Cryptococcosis in children in the state of Pará, Brazil. Rev Soc Bras Med Trop. 1999;32(5):505–8.
28. Darzé C, Lucena R, Gomes I, Melo A. Clinical and laboratory characteristics of 104 cryptococcus meningoencephalitis cases. Rev Soc Bras Med Trop. 2000;33(1):21–6.
29. Mora DJ, Colombo ERC, Ferreira-Paim K, Andrade-Silva LE, Nascentes GAN, Silva-Vergara ML. Clinical, epidemiological and outcome features of patients with cryptococcosis in Uberaba, Minas Gerais, Brazil. Mycopathologia. 2012; doi:10.1007/s11046-011-9504-9.
30. Yuchong C, Fubin C, Jianghan C, Fenglian W, Nan X, Minghui Y, et al. Cryptococcosis in China (1985–2010): review of cases from Chinese database. Mycopathologia. 2012; doi:10.1007/s11046-011-9471-1.
31. Favalessa OC, Ribeiro LC, Tadano T, Fontes CJF, Dias FB, Coelho BPA, et al. First description of phenotypic profile and in vitro drug susceptibility of *Cryptococcus* spp. yeast isolated from HIV-positive and HIV-negative patients in State of Mato Grosso. Rev Soc Bras Med Trop. 2009;42(6):661–5.
32. Mondon P, Petter R, Amalfitano G, Luzzati R, Concia E, Polacheck I, et al. Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans*. Antimicrob Agents Chemother. 1999;43(8):1856–61.
33. Silva PR, Rabelo RAS, Terra APS, Teixeira DNS. Susceptibility to antifungal agents among *Cryptococcus neoformans* varieties isolated from patients at a university hospital. Rev Soc Bras Med Trop. 2008;41(2):158–62.