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Original article

Characterization of clinical isolates of the *Cryptococcus neoformans-Cryptococcus gattii* species complex from the Amazonas State in Brazil

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ABSTRACT

Background: The differentiation and classification of pathogenic *Cryptococcus* species provides useful data for epidemiological studies and for the clinical diagnosis and treatment of patients.
Aims: The aim of this study was to characterise 40 clinical *Cryptococcus* isolates obtained from patients at the Tropical Medicine Foundation of Amazonas (FMTAM) from 2006 to 2008.
Methods: It was used phenotypic (i.e., enzyme production and antifungal resistance) and molecular biological (*URA5*-RFLP) experiments.
Results: Patients with HIV/AIDS were most affected with cryptococcosis. Thirty-one (75.5%) of the clinical isolates were classified as *Cryptococcus neoformans* and 9 (22.5%) as *Cryptococcus gattii*. High amounts of protease and phospholipase enzymes were produced by most of the isolates. Using the disk diffusion test (CLSI M44-A), 81, 35 and 100% of the *C. neoformans* isolates were characterized as susceptible to fluconazole, itraconazole and amphotericin B, respectively, whereas 78, 56 and 100% of the *C. gattii* isolates were susceptible to these antimicrobial agents. The average of Minimal Inhibitory Concentration (MIC) for *C. neoformans* and *C. gattii* isolates was 0.26 and 0.58 μg/mL, respectively. The 9 isolates of *C. gattii* had a fingerprint pattern comparable with the VGII molecular type, while all 31 isolates of *C. neoformans* presented with a pattern consistent with the VNI type.
Conclusions: This study confirms the importance of HIV/AIDS for the cryptococcosis epidemiology, the susceptibility of the isolates to amphotericin B and the high prevalence of the molecular genotypes VNI and VGII in the north of Brazil.

Caracterización de aislamientos clínicos del complejo *Cryptococcus neoformans-Cryptococcus gattii*, del Estado Amazonas - Brasil

Palabras clave:
Cryptococcus neoformans
Cryptococcus gattii
Epidemiología
Fenotipo y genotipo

RESUMEN

Antecedentes: La diferenciación y clasificación de las especies patógenas del género *Cryptococcus* aporta datos importantes para la asistencia clínica y para estudios epidemiológicos.
Objetivos: El objetivo de este trabajo fue caracterizar 40 aislamientos clínicos del complejo *Cryptococcus neoformans* de pacientes que fueron atendidos en la Fundación de Medicina Tropical de Amazonas desde 2006 hasta 2008.
Métodos: Se utilizaron métodos fenotípicos (producción de enzimas y pruebas de sensibilidad a los antifúngicos) y moleculares (URA5-RFLP).
Resultados: Los pacientes con VIH/sida fueron los más afectados de criptococosis. Se observó que 31 (75,5%) y 9 (22,5%) de los aislamientos fueron *Cryptococcus neoformans* y *Cryptococcus gattii*, respectivamente. La producción de proteasa y fosfolipasa fue alta en la mayoría de las cepas. Utilizando la prueba de difusión en disco (CLSI M44-A) se observó que el 81, 35 y 100% de los aislamientos de *C. neoformans* fueron sensibles al fluconazol, itraconazol y amphotericin B, respectivamente, mientras que 78, 56 y 100% de los aislamientos de *C. gattii* fueron sensibles a

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estas sustancias. El valor promedio de la concentración mínima inhibitoria (CMI) para *C. neoformans* y *C. gattii* fue de 0,26 y 0,58 mg/ml, respectivamente. Todos los aislamientos (9) de *C. gattii* presentaron un patrón de electroforesis compatible con el genotipo VGII, y todos los aislamientos (31) de *C. neoformans* presentaron el genotipo VNI.

Conclusiones: Este estudio confirma la importancia del HIV/sida para la epidemiología de la criptococosis, la sensibilidad de los aislamientos a la anfotericina B y la alta prevalencia de los genotipos moleculares VNI y VGII en el norte de Brasil.

Cryptococcosis is an opportunistic fungal disease caused by the encapsulated yeast species *Cryptococcus neoformans* and *Cryptococcus gattii*.[17] The infection is acquired by the inhalation of desiccated yeasts or spores and can develop to the brain or other systemic organs.[18] Infection with *Cryptococcus* spp. is the major cause of fungal meningitis in immunocompromised patients, resulting in elevated morbidity and mortality rates.[21] Furthermore, immunocompromised status is the most common risk factor for infection with *C. neoformans*. Additionally, there are increasing numbers of immunocompetent patients with fungal meningitis who are infected with *C. gattii*.[21,32] The preferred treatment for cryptococcal meningitis is amphotericin B, but a side effect of the drug manifested by nephrotoxicity limits its clinical use and increases the importance of antifungal susceptibility testing.[10,12]

Based on serological studies, using antibodies against the fungal capsule, seven serotypes of *Cryptococcus* have been identified: A, B, C, D, AB, AD and BD.[3,5,20] *C. neoformans* serotypes A, D and AD are found in cities, whereas the *C. gattii* serotypes B and C are predominately localised in tropical and subtropical regions.[1,13,19] In 1999 an outbreak of *C. gattii* occurred in Canada, raising the possibility that this species could be localised in both tropical and temperate climates.[11]

In addition to the serotypes of *C. neoformans* and *C. gatti*, molecular analyses, including M13 fingerprinting, *URA5*-RFLP (Restriction Fragment Length Polymorphism) and AFLP (Amplified Fragment Length Polymorphism), have identified 8 molecular genotypes: VNI and VNII (Serotype A); VNIII (Hybrid AD); VNIV (Serotype D); and VGI, VGII, VGIII and VGIV (Serotypes B and C).[17] Molecular genotyping contributes to better and more accurate clinical diagnoses and characterizes genetic diversity for global epidemiological studies.[24]

The aim of this work was to characterize forty *Cryptococcus* isolates from patients at the Tropical Medicine Foundation of Amazonas (FMTAM) during 2006–2008 by using phenotypic and molecular biological experiments.

1. Materials and methods

1.1. Isolates and strains

Forty isolates were obtained from patients with cryptococcal infection who were hospitalized in FMTAM between March 2006 and February 2008. A single sample was collected from each patient and stored at 4 °C in the FMTAM fungal collection. They were grown on Sabouraud agar at 30 °C for 48 h. For molecular genotyping, the following standard strains were used: WM 148 (serotype A,VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), WM 629 (serotype D, VNIV), WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 161 (serotype B, VGIII) and WM 779 (serotype C, VGIV). These reference strains were kindly provided by the fungal collection at FIOCRUZ-Rio de Janeiro-Brazil. Also, the reference strains WM 148 and WM 178 were used as reference strains for L-canavanine glycine bromothymol blue (CGB) species determination, enzyme production and susceptibility assays using antifungal agents.

1.2. Patient information

The epidemiological profile of the patients (age, sex, immunological status and residence) was obtained by analysis of the examination requisition.

1.3. Identification of the species

CGB agar was utilized to differentiate the species *C. neoformans* and *C. gattii* as previously described.[13] *C. gattii* strains use the glycine in the media as a source of carbon and nitrogen, are resistant to canavanine and produce a blue cobalt colour during incubation, whereas *C. neoformans* do not exhibit any colour change in the media.

1.4. Enzyme quantification

The production of proteinases and phospholipases were measured as previously described.[23,25,33] The fungal isolates were inoculated at equidistant points on plates of proteinase agar and phospholipase agar, and the enzyme activity (Pz) was calculated using the relation between the diameter of the colony (dc) and the diameter of the colony and precipitation zone (dcp). The results were classified as negative ($Pz = 1$), positive ($0.64 \geq Pz < 1$) or strongly positive ($Pz < 0.64$).

1.5. Susceptibility to antifungal agents

Susceptibility tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) document M44-A.[4] Disks impregnated with fluconazole (25 μg), itraconazole (10 μg) or amphotericin B (100 μg) (Cecon-Sensifungidisc, São Paulo, Brazil) were used in the assays. The diameter of the inhibition haloes was used to determine the susceptibility of the yeast to the antifungal compounds. Cut-off values were as follows: Amphotericin B > 10 mm – susceptible (S), <10 mm –resistant (R); itraconazol >20 mm – S, 19–12 mm – intermediate (I), <1 mm – R and fluconazole >19 mm – S; 19–14 mm – I; <14 mm – R. The determination of the MIC for amphotericin B was performed using the *E*-test-AB BIODISK method as described previously.[14]

1.6. Characterization of molecular genotypes

The genotypic characterization was carried out as described previously.[17] Fungal DNA was extracted with the QIAamp tissue kit (Qiagen, Germany) according to the manufacturer's protocol for DNA extraction from yeast, including digestion with lyticase and RNAase. PCR of the *URA5* gene was conducted in a final volume of 50 μL, and each reaction contained 50 ng of DNA, 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$); 0.2 mM each of dATP, dCTP, dGTP and dTTP; 3 mM magnesium acetate; 1.5 U AmpliTaq DNA polymerase (Invitrogen, Carlsbad, California) and 50 ng of each primer *URA5* (5′ATGTCCTCCCAAGCCCTCGACTCCG3′) and SJ01 (5′TTAAGACCTCTGAACACCGTACTC3′). The PCR was

Table 1
Epidemiological characteristics of patients with cryptococcosis.

	C. neoformans	*C. gattii*	Total (%)
Sex			
Female	9	3	12 (30)
Male	22	6	28 (70)
Age (Years)			
0– 5	0	3	3 (8)
16–30	18	3	21 (53)
31–45	8	2	10 (25)
46–60	3	1	4 (10)
>60	1	1	2 (5)
HIV Infection			
Negative	0	4	4 (10)
Positive	24	5	29 (73)
Unknown	7	0	7 (18)
Area of the residence in Manaus			
North	4	1	5 (12)
South	2	1	3 (8)
East	8	2	10 (24)
West	1	0	1 (2)
South Central	2	1	3 (8)
Midwest	4	1	5 (12)
Unknown	10	3	13 (33)
Biological sample			
Cerebrospinal fluid	29	8	37 (92)
Blood culture	2	0	2 (5)
Sputum	0	1	1 (3)

performed in a PerkinElmer thermal cycler (model 480) for 35 cycles of a 2 min initial denaturation at 94 °C, 45 s denaturation at 94 °C, 1 min annealing at 61 °C and 2 min extension at 72 °C, followed by a final extension cycle for 10 min at 72 °C. The PCR products were double digested with *Sau*96I (10 U/μL) and *Hha*I (20 U/μL) for 3 h or overnight and separated by 3% agarose gel electrophoresis at 100 V for 5 h.

2. Results

The epidemiological characteristics of the patients with cryptococcosis were evaluated. Males were more commonly infected (70%) than females, and the age group most affected was between 16 and 30 years (53%; median = 28.5 years) (Table 1). The majority of patients with cryptococcosis were also infected with HIV (72%) and resided in the east zone of the city of Manaus (25%) (Table 1). Cerebrospinal fluid (CSF) was collected for the isolation of the fungal agents from most patients (93%). Isolates were inoculated on a CGB medium; 9 were positive for the presence of *C. gattii*, and 31 were positive for *C. neoformans*.

All of the isolates had high protease production (Pz < 0.64) and an average Pz = 0.3 for both *Cryptococcus* species. All but 1 isolate was positive for the production of phospholipase (average Pz = 0.53). Twenty-six *C. neoformans* isolates (83.8%) presented strong positive results (Pz < 0.64), and 4 isolates (12.9%) presented positive results (Pz ≥ 0.64 and < 1.0), whereas all 9 isolates of *C. gattii* were classified as strong-positive producers of phospholipase.

Using the disk diffusion test (CLSI M44-A), 81, 35 and 100% of the isolates of *C. neoformans* were identified as susceptible to fluconazole, itraconazole and amphotericin B, respectively, and 78%, 56% and 100% of the isolates of *C. gattii* were susceptible to these antifungal compounds (Table 2). The average MIC for amphotericin B, evaluated by *E*-test methodology, was 0.26 ± 0.22 μg/mL for *C. neoformans* and 0.58 ± 0.53 μg/mL for *C. gattii*.

DNA was extracted from all clinical isolates and was used to identify the molecular genotypes by *URA5*-RFLP. All 9 isolates of *C. gattii* possessed a fingerprint pattern consistent with the VGII molecular genotype, and all 31 isolates of *C. neoformans* had the profile of the VNI molecular type.

Table 2
- Susceptibility of *C. neoformans* and *C. gattii* to antifungal compounds, as measured by disk diffusion.

	Antifungal	Susceptible	Intermediate	Resistant
		N (%)	N (%)	N (%)
C. neoformans	Fluconazole	25 (80.6)	5 (16.1)	1 (3.3)
	Itraconazole	11 (35.4)	20 (64.6)	0
	Amphotericin B	31 (100)	0	0
C. gattii	Fluconazole	7 (77.7)	0	2 (22.3)
	Itraconazole	5 (55.5)	4 (44.5)	0
	Amphotericin B	9 (100)	0	0

3. Discussion

The total number of the inhabitants in the state of Amazonas is approximately 3.5×10^6 and according to the Mycology Service of the Foundation of Tropical Medicine of Manaus, 20–25 new cases of cryptococcosis occur annually in the region. Manaus, the capital of Amazonas State, is located near the centre of the Amazon Basin on the left bank of the Rio Negro, consisting of Tertiary sediments on sandy-clay land. The region has an average rainfall of 2300 mm/year and is thus associated with a hot and humid climate and lush vegetation.[31] In this study, the incidence of cryptococcosis was not related to these weather patterns, as the number of isolates was similar throughout the year. The identification of potential environmental sources of *C. neoformans* and *C. gatii* that are introduced into the human population requires further investigation. It is hypothesise that the sources of contamination are similar to those described in other countries: human infection with the *Cryptoccocus* type VNI primarily occurs from contact with bird faeces, and infection with the VGII type usually occurs following contact with decomposing vegetal biomass.

This is one of the first studies to characterise cryptococcosis in the Amazonas State in Brazil. This study determined that the patients most affected by cryptococcosis were male, young (16–30 years old), HIV-positive and inhabitants of the east zone of the city of Manaus.

The number of cases of HIV/AIDS reported annually in the Amazonas State is approximately 700, half of them receive HAART treatment.[30] AIDS is the most important risk factor for cryptococcosis, due to the suppression of immune responses by the HIV virus.[9,13,26] *C. neoformans* was the species most frequently isolated (n = 31; 77.5%), which is consistent with the 82.3% reported in the literature.[17,19] *C. neoformans* almost exclusively causes disease in immunocompromised patients, and the majority of the isolates that we obtained came from patients with AIDS.[17,19] Among the nine *C. gattii* isolates, three were obtained from immunocompetent patients, four from AIDS patients and two from patients without documented immune status.

In this study, we obtained three isolates from children aged 0–15 years. *C. gattii* was the primary causative agent of meningitis in these patients. In a previous study in the Amazonas State from 1988 to 1998, Martins[15] reported that the frequency of cryptococcosis in children represented 33% of the total cases. In the Pará state, neighbouring the Amazonas State, 19–24% of cryptococcosis cases were reported in children over the last 10 years.[28,29] These studies demonstrate that in the Amazon Rainforest, the prevalence of cryptococcosis in children is high and is also associated with the molecular type VGII. Further epidemiological studies and environmental sampling are necessary to define the importance of the environmental conditions in the incidence of cryptococcosis cases in children.

The production of lipases and proteases is related to the virulence of *Cryptococcus* pathogenic species.[7,8] These enzymes are involved in the destruction of cellular structures to both obtain nutrients for the fungal pathogens and to facilitate spread

throughout tissues. Both *C. neoformans* and *C. gattii* produced proteases and lipases in similar quantities, and no difference was observed between molecular types.

Antifungal cryptococcosis testing showed 80% and 77% susceptibility to fluconazole in *C. neoformans* and *C. gattii*, respectively. This maintenance drug is commonly used in patients with AIDS to prevent opportunistic fungal diseases, and the emergence of resistant strains has been described.[6,9] *C. neoformans* and *C. gattii* had 35% and 55% susceptibility to itraconozole, respectively, and all of the clinical isolates were susceptible to amphotericin B. These results are consistent with previous studies.[14,22,27] Amphotericin B is considered to be the gold standard for cryptococcosis treatment.[2] In one study, amphotericin B resistance was reported in 10% of clinical isolates,[5] but other studies demonstrate low in vitro resistance to this compound.[14,27] Although all isolates were considered to be susceptible to amphotericin B (MIC < 2 μg/mL), the average MIC of the *C. gattii* isolates (0.56 μg/mL) was greater than that of *C. neoformans* isolates (0.26 μg/mL). This result has been previously reported[14] and highlights the potential for differential efficacy of therapeutics in the treatment of *C. neoformans* and *C. gattii*.

The identification of pathogenic *Cryptococcus* species and subspecies are important for both clinical guiding and as well as for epidemiology. Recently, false-positive *C. gattii* results have been reported using the conventional CGB differentiation method due to the existence of *C. neoformans* isolates that are resistant to canavanine.[13] Meanwhile, PCR fingerprinting and PCR-RFLP are being utilized more frequently.[9,20,24] In the current study, PCR-RFLP was used to characterize the *Cryptococcus* isolates as the molecular types VNI and VGII. Furthermore, these results are consistent with previous studies that identified *C. neoformans* VNI as the primary agent of cryptococcosis.[13,16,19] A study that characterized 63 Brazilian isolates described the prevalence of the molecular types VNI (82.3%), VGII (13.6%) and VNII (3.0%),[17] and more recent studies in the Pará state reinforce the prevalence of VNI and VGII as being the principal molecular types of *C. neoformans* and *C. gattii*, respectively, in the northern region of Brazil.[17]

Data from this study can assist with current and future management of cryptococcosis in Northern Brazil by identifying the frequency of *C. neoformans* and *C. gattii* in patients as well as their susceptibility to commonly used antifungal drugs. These data also contribute to epidemiological studies of the distribution of different molecular types of *Cryptococcus* species in the Brazilian Amazon.

Conflict of interest

The authors have no conflict of interest to declare.

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References

1. Bernardo FM, Martins HM, Martins ML. Urban sources of *Cryptococcus spp* – Lisbon. Ver Port Clin. 2001;96:157–60.
2. Brasil, Ministério da Saúde. Documentos e publicações em DST e AIDS. Coordenação do programa nacional de DTS/AIDS. *Epidemiol Vigilance*. 2004:1. Available at: http://www.aids.gov.br [consulted on 03-22-2009].
3. Bovers M, Hagen F, Boekhout T. Diversity of the *Cryptococcus neoformans–Cryptococcus gattii* species complex. Rev Iberoam Micol. 2008;25:4–12.
4. Clinical Laboratory Standards Institute (CLSI). Reference method for antifungal disk diffusion susceptibility testing of yeasts; Approved guideline. In: NCCLS document M44-A. Wayne, PA: National Committee for Clinical Laboratory Standards; 2004.
5. De Bedout C, Ordóñez N, Gómez BL, Rodríguez MC, Arango M, Restrepo A, et al. In vitro antifungal susceptibility of clinical isolates of *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Rev Iberoam Micol. 1999;16:36–9.
6. Espinel-Ingroff A. Comparison of three commercial assays and a modified disk diffusion assay with two broth microdilution reference assays for testing zygomycetes, *Aspergillus* spp., *Candida* spp., and *Cryptococcus neoformans* with posaconazole and amphotericin B. J Clin Microbiol. 2006;44:3616–22.
7. Henry J, Guillotte A, Luberto C, Del Poeta M. Characterization of inositol phosphosphingolipid-phospholipase C1 (Isc1) in *Cryptococcus neoformans* reveals unique biochemical features. FEBS Lett. 2011;585:635–40.
8. Heung LJ, Kaiser AC, Luberto C, Poeta MD. The role and mechanism of diacylglycerol-protein kinase C1 signaling in melanogenesis by *Cryptococcus neoformans*. J Biol Chem. 2005;280:28547–55.
9. Jain N, Wickes BL, Keller SM, Fu J, Casadevall A, Jain P, et al. Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India. J Clin Microbiol. 2005;43:5733–42.
10. John RP, Gary MC. Drug resistance in *Cryptococcus neoformans*. J Clin Microbiol. 1999;2:259–69.
11. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, et al. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci USA. 2004;172:58–63.
12. Kotwani RN, Gokhale PC, Bodhe PV, Kirodian BG, Kshirsagar NA. Safety and efficacy of liposomal amphotericin B in patients with cryptococcal meningitis. J Assoc Physicians India. 2001;49:1086–90.
13. Kwon-Chung KJ, Sorrell TC, Dromer F, Fung E, Levitz SM. Cryptococcosis: clinical and biological aspects. Med Mycol. 2000;38:205–13.
14. 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;36:2817–22.
15. Martins LMS. Epidemiologia da criptococose em crianças e adultos jovens e diversidade de *Cryptococcus neoformans* no meio Norte do Brasil. MSc thesis. Fiocruz, Rio de Janeiro: Instituto Oswaldo Cruz; 2003.
16. Matsumoto MT, Fusco-Almeida AM, Baeza LC, Melhem MSC, Medes-Giannini MJS. Genotipagem, sorotipagem e determinação de mating-type de isolados clínicos de *Cryptococcus neoformans* do Estado de São Paulo, Brasil. Rev Inst Med Trop S Paulo. 2007;39:3–6.
17. Meyer WE, Kidd S, Castañeda A, Jackson S, Huynh M, Latouche GN. Molecular typing of Ibero American *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003;9:189–95.
18. Moreira TA, Ferreira MS, Ribas RM, Borges AS. Criptococose: estudo clínico-epidemiológico, laboratorial e das variedades do fungo em 96 pacientes. Rev Soc Bras Med Trop. 2006;39:255–8.
19. Nishikawa MM, Lazera SM, Barbosa GG, Trilles L, Balassiano BR, Macedo RCL, et al. Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and environmental sources in Brazil: analysis of host and regional patterns. J Clin Microbiol. 2003;41:73–7.
20. Ohkusu M, Hata K, Takeo K. Serotype, mating type and ploidy of *Cryptococcus neoformans* strains isolated from patients in Brazil. Rev Inst Med Trop S Paulo. 2002;44:299–302.
21. Pappalardo MCSM, Melhem MSC. Criptococose: revisão sobre a experiência brasileira sobre a doença. Rev Inst Med Trop S Paulo. 2003;45:73–8.
22. Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ. In vitro susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species to itraconazole: global survey of 9359 isolates tested by clinical and laboratory standards institute broth microdilution methods. J Clin Microbiol. 2005;43:3807–10.
23. Price MF, Wilkinson ID, Gentry IO. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia. 1982;20:15–20.
24. Ribeiro MA, Ngamskulrungroj P. Molecular characterization of environmental *Cryptococcus neoformans* isolated in Vitoria, ES, Brazil. Rev Inst Med Trop S Paulo. 2008;50:25–8.
25. Ruchel R, Uhlemann K. A comparison of secretory proteinases from different strains of *Candida albicans*. Sabouraudia. 1982;20:233–44.
26. Rude TH, Toffaletti DL, Cox GM, Perfect JR. Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*. Infect Immun. 2002;70:5684–94.
27. Salgal G, et al. Unusual presentation of central nervous system Cryptococcal infection in an immunocompetent patient. Am J Neuroradiol. 2005;26:2522–6.
28. Santos WR, Meyer W, Wanke B, Costa SPSC, Luciana T, Nascimento JLM, et al. Primary endemic *Cryptococcosis gattii* by molecular type VGII in the state of Pará, Brazil. Mem Inst Oswaldo Cruz. 2008;103:813–8.
29. Santos WR, Meyer W, Wanke B, Costa SPSC, Luciana T, Nascimento JLM, Medeiros R. Caracterização dos isolados clínicos de espécies do complexo Cryptococcus neoformans em Belém do Pará. In: Norte do Brasil. Congresso Brasileiro de Micologia; 2007.
30. Silva LCF, Santos EM, Silva NAL, Miranda AE, Talhari S, Toledo LM. Padrão da infecção pelo HIV/AIDS em Manaus, Estado do Amazonas, no período de 1986 a 2000. Rev Soc Bras Med Trop. 2009;42:543–50.
31. Silva ML. Silva MSR Perfil da qualidade das águas subterrâneas de Manaus. Holos Environ. 2007;1:1–13.
32. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. Med Mycol. 2001;39:155–68.
33. Vidotto V, Melhem M, Pukinskas S, Aoki S, Carrara C, Pugliese A. Extracellular enzymatic activity and serotype of *Cryptococcus neoformans* strains isolated from AIDS patients in Brazil. Rev Iberoam Micol. 2005;22:29–33.