Primary endemic Cryptococcosis gattii by molecular type VGII in the state of Pará, Brazil

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In order to study the infectious agents causing human disseminated cryptococcosis in the state of Pará, North Brazil, 56 isolates of Cryptococcus *spp. (54 isolated from cerebral spinal fluid and two from blood cultures) from 43 cases diagnosed between 2003-2007 were analysed. The species were determined through morphological and physiological tests and genotypes were determined by* URA5*-RFLP and PCR-fingerprinting (wild-type phage M13). The following species and genotypes were identified:* Cryptococcus neoformans *VNI (28/56, 50%),* Cryptococcus gattii *VGII (25/56, 44.64%) and* C. gattii *VGI (3/56, 5.26%). The genotype VNI occurred in 12 out of 14 HIV-positive adults, whereas the genotype VGII occurred in 11 out of 21 HIV-negative adults ($p < 0.02$, OR = 6.6 $IC_{95\%}$ 0.98-56.0). All patients less than 12 years old were HIV negative and six cases were caused by the VGII genotype, one by the VGI and one by VNI. Therefore, endemic primary mycosis in HIV-negative individuals, including an unexpectedly high number of children, caused by the VGII genotype deserves further study and suggests the need for surveillance on cryptococcal infection in the state of Pará, Eastern Amazon.*

Key words: *Cryptococcus gattii* - VGII genotype - endemic mycosis - children - state of Pará

Cryptococcosis is a significant life-threatening fungal infection that affects humans and a large variety of animals. It is caused by two species of *Cryptococcus*, *C. neoformans* (serotypes A, D and the hybrid AD) and *C. gattii* (serotypes B and C). *C. gattii*, previously regarded as a variety of *C. neoformans*, is now recognized as a distinct species (Kwon-Chung & Varma 2006).

C. neoformans-induced cryptococcosis occurs worldwide and is an important cause of morbidity and mortality in immunocompromised individuals, especially AIDS patients. Cryptococcosis caused by *C. gattii*, however, is mainly seen in non-immunocompromised patients (Lazera et al. 2005) and regarding its geographical distribution, it is considered a tropical pathogen. However, the recent description of the Vancouver Island (Canada) outbreak suggests that *C. gattii* has adapted to more temperate environments (Kidd et al. 2004).

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The infections caused by *C. neoformans* and *C. gattii*, as well as their corresponding serotypes and molecular types, present with different clinical symptoms and have a diverse prognosis. Therefore, an understanding of the prevailing species and types in specific geographic regions is important. The mycosis caused by *C. neoformans* serotype A is widespread and predominantly infects immunocompromised individuals. However, *C. neoformans* serotype D is predominantly found in Europe and mainly infects elderly subjects, although it has been rarely detected in Brazil (Dromer et al. 1996, Nishikawa et al. 2003).

Cryptococcosis caused by *C. gattii* (mainly serotype B) has been predominantly detected in immunocompetent patients and presents as a regressive lung lesion which is usually undetected, or as a peripheral lung nodule which has to be differentiated from a malignant tumour. Both species cause a basal meningoencephalitis, with or without lung lesions, that usually causes an increased intracranial pressure, cranial nerve damage and hydrocephalus. In HIV-negative patients the infection may also cause blindness and hearing loss. Cryptococcosis caused by *C. gattii* typically evolves sub-acutely or chronically and is often confused with viral or bacterial meningoencephalitis or other infections, including tuberculosis (Lazera et al. 2005, Lin & Heitman 2006).

C. neoformans serotype A is ubiquitous and has been associated with organic material in pigeon habitats, captive birds in domestic environments, domestic dust and

wood decay in hollow trees of different species (Passoni et al. 1998, Nishikawa et al. 2003). In Brazil, it is the most prevalent serotype in clinical (89%) and environmental isolates (90.2%) in the South, Southeast and Central West regions, whereas *C. gattii* predominates in the Northeast region in clinical and environmental isolates (64%) (Nishikawa et al. 2003).

Most clinical isolates are haploid and heterothallic but some may be aneuploid or even diploid. The mating type of *Cryptococcus* is determined by a one-locus, two allele system (MAT α and MAT a) that produces viable progeny in vitro in a 1:1 ratio. Clinical and environmental isolates are predominantly MAT α, which has been suggested to be of higher virulence in animal models (Kwon-Chung & Bennett 1992).

PCR fingerprinting is being used in ongoing global surveys to study the epidemiology of cryptococcosis. By using primers for the minisatellite-specific core sequence of the wild-type phage M13, in combination with *URA5*-RFLP analysis, eight major molecular types (VN I-IV for *C. neoformans* and VG I-IV for *C. gattii*) have been identified (Meyer et al. 2003). The corresponding genotypes of these molecular types were identified using amplified fragment length polymorphism PCR according to Boekhout et al. (2001).

In the Southeast and South regions of Brazil, where consistent data are available, human cryptococcosis is caused predominantly by *C. neoformans* serotype A, VNI and is associated with AIDS (Casali et al. 2003, Igreja et al. 2004). However, the few studies performed in the Northeast region suggest epidemiological differences, particularly the occurrence of meningitis by *C. gattii* serotype B in native adults and children in this region (Lazera et al. 2005). Additionally, one study performed in the North region, in Belém do Pará, also describes cryptococcosis in immunocompetent children (Corrêa et al. 1999). To increase the understanding of cryptococcosis in the North region of Brazil, we performed a retrospective and prospective study of isolates obtained from patients with meningitis, identifying the species, serotypes and molecular types of *Cryptococcus*. Clinical and epidemiological data were also analysed providing an epidemiological view on cryptococcosis and its causative agents in the Eastern Amazon.

MATERIALS AND METHODS

Cryptococcus identification - From May 2003-April 2007, isolates of *Cryptococcus* spp. isolated from patients admitted to the João Barros Barreto Hospital, a reference centre in the city of Belém, were identified by morphological and physiological tests, including phenol oxidase production on niger seed agar (NSA), cycloheximide sensitivity, assimilation of carbon and nitrogen sources (Vitek ICB, bioMerieux, Durham, USA) and growth in canavanine-glycine-bromothymol blue (CGB) media. Additionally, the Crypto-Chek Kit (Iatron Laboratories, Tokyo, Japan) was used to discriminate between different serotypes of the same species.

Statistical analysis - The clinical and fungal data from the isolates were analysed with the chi-square test (Yates correction). All p-values less than 0.05 were considered significant.

Reference strains - The following set of standard laboratory strains, representing each molecular type, were used as controls in PCR-fingerprinting and *URA5*-RFLP: 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).

DNA extraction - High molecular weight DNA was extracted according to Ferrer et al. (2001). Half an inoculation loop of culture was frozen at -20°C for 1 h and then incubated at 65°C for 1 h in 0.5 mL of extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). The lysate was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v). DNA was recovered by isopropanol precipitation, washed with 70% (v:v) ethanol and diluted in sterile water.

URA5-RFLP - PCR of the *URA5* gene was conducted in a final volume of 50 µL. Each reaction contained 50 ng of DNA, 1X PCR buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 2 mM DCT, 50% glycerol, 0.1% Tween-20), 0.2 mM of each dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH), 2 mM magnesium chloride, 2.5 U Taq DNA polymerase (Bioline) and 25 ng of each primer URA5 (5' ATGTCCTCCCAAGCCCTCGACTCCG 3') and SJ01 (5' TTAAGACCTCTGAACACCGTACTC 3'). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) using the following parameters; 94°C for 5 min initial denaturation, 35 cycles denaturation at 94°C for 45 s, 1 min annealing at 63°C and 2 min extension at 72°C, followed by a final extension cycle for 10 min at 72°C. PCR products were double digested with Sau96I (10 U/µL) and HhaI (20 U/µl) for 3 h and the fragments were separated on a 3% agarose gel stained with ethidium bromide. RFLP patterns were assigned visually by comparison with the patterns obtained from the reference strains described above.

PCR-fingerprinting - PCR-fingerprinting reactions were conducted in a volume of 50 µL containing 25 ng genomic DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM magnesium acetate, 30 ng primer (5' GAGGGTGGCGGTTCT 3'), and 2.5 U Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) with 20 s of denaturation at 94°C, 1 min annealing at 50°C and 20 s extension at 72°C, followed by a final extension cycle for 6 min at 72°C. Amplification products were concentrated to approximately 15 µL and separated by electrophoresis on 1.4% agarose gels stained with ethidium bromide (Meyer et al. 2003). PCR-fingerprinting profiles were visually compared to the reference strains to determine the molecular types. The genetic relationships of the isolates were analysed using the 1D gel analysis module (BioGalaxy [BioAware, Han-

nut, Belgium]) in BioloMICS version 7.5.30 (BioAware). Similarity coefficients were calculated by using the Dice algorithm and cluster analyses were performed with the Unweighted Pair Group Method with Arithmetic mean.

Molecular determination of the mating type - The MAT α and MAT a pheromones were determined in all isolates by PCR according to Chaturvedi et al. (2000), using the α-mating type-specific primers 5' - CTTCACTGCCATCTTCACCA-3' and 5' -GACACAAAGGGTCATGCCA-3', and the a-mating type-specific primers 5' - CGC CTT CAC TGC TAC CTT CT-3' and 5' -AAC GCA AGA GTA AGT CGG GC-3'. *C. gattii* isolate mating types were determined using the primers MFA2U (5' ACACCGCCTGTTACAATGGA 3') and MFA2L (5' CAGCGTTTGAAGATGGACTTT 3'), as described by Fraser et al. (2003).

RESULTS

A total of 56 cryptococcal isolates from 43 patients with meningitis were analysed. The majority of these isolates (54 of 56) were isolated from the cerebral spinal fluid of patients, while only two were isolated from blood cultures. A single isolate was obtained from 31 patients; two isolates were obtained from 11 patients and three from one patient. In this cohort, 26 (60.5%) patients were male and 17 (39.5%) female. Eight (18.6%) children (< 12 years old) were all HIV negative and the remaining 35 (81.4%) patients comprised 21 (60%) HIV-negative and 14 (40%) HIV positive patients.

C. gattii was more frequently isolated from HIV-negative patients (19/29; 65.5%) and *C. neoformans* was predominantly isolated from the HIV-positive patients (12/14, 85.7%; $p < 0.005$; OR = 11.4; $IC_{95\%}$ 1.8-92.2). The remaining two HIV-positive patients had *C. gattii*-induced cryptococcosis (Table). The identification of the species of *Cryptococcus* by the CGB test demonstrated that of the 56 isolates, 27 (48.2%) were *C. neoformans*, 20 (35.7%) were *C. gattii* and nine produced ambiguous results [16.1 % (Table)]. Based on the results of the serotyping analysis, 21 isolates were serotype B, 20 were serotype A and 15 were indeterminate (7 *C. gattii* and 8 *C. neoformans*). Five isolates obtained from two children from the Northeast nesoregion of Pará produced indeterminate results in both the serotyping and CGB tests. Three molecular types were identified by *URA5*-RFLP and PCR-fingerprinting: VNI (28/56, 50%), VGII (25/56, 44.6%) and VGI [3/56, 5.4% (Table)]. The VNI genotype was isolated from 12 of 14 HIV-positive adults, whereas the VGII genotype was isolated from 11 of 21 HIV-negative adults ($p < 0.02$, OR = 6.6 $IC_{95\%}$ 0.98-56.0). All isolates were identified as MAT α using mating type-specific primers (Fraser et al. 2003, Holliday et al. 2003). The geographical origin of the 43 cases based on molecular type and mesoregion of the state of Pará is shown in Figure.

DISCUSSION

The North region of Brazil encompasses a large area of the Amazon rainforest and more studies on human cryptococcosis and the molecular profiles of the infecting strain(s) need to be performed. A study on cryptococcosis in children in the state of Pará described 19 cases, from which nine isolates were determined to be *C. gattii* (Corrêa et al. 1999). In the present study, the incidence of *C. gattii*-induced meningitis is similar to the previously described cases in the Northeast region (Lazera et al. 2005). However, it is distinct from the cases described in the South and Southeast regions of Brazil (Nishikawa et al. 2003).

In the state of Pará, *C. gattii* was the main causative agent of meningitis in the HIV-negative patients and occurred also in two HIV-positive patients. The high frequency of cryptococcal meningitis in HIV-negative children (8/43; 18.6%) observed from 2003-2007 supports previous studies by Corrêa et al. (1999) and suggests that cryptococcal infection occurs early in life in this region. Previous studies have demonstrated that infantile cryptococcosis accounts for a significant fraction of cryptococcosis cases in the neighbouring state of Amazonas (33%, n = 75) (Santos 2000) and the Piauí and Maranhão, states of the Northeast region (21%, n = 257) (Martins 2003). Since the present study is based on isolates obtained from patients representing only part of the cases, the real occurrence of this mycosis is largely underestimated.

We were unable to identify the *Cryptococcus* species in a significant number of isolates using the CGB test. Interfering factors, such as contamination or excessive inoculum, were excluded by re-plating on NSA medium and carefully repeating the test, which yielded the same results. Similarly, a significant number of isolates were untypable by serotyping. These results demonstrate the occurrence of atypical phenotypes among clinical isolates in the state of Pará, which may cause difficulties in their species identification at the local public laboratories. Usually most *Cryptococcus* isolates are haploid, nevertheless some diploid or aneuploid isolates may lead to unstable phenotypes, which may be recombinants or hybrids (Kwon-Chung & Varma 2006). Further genetic studies are necessary to evaluate the atypical clinical isolates from Pará.

The molecular type VGII was found to be the main causative agent in cases of *C. gattii*-induced cryptococcosis. These findings are in agreement with a recent Brazilian study demonstrating that the VGII genotype is the main cause of endemic cryptococcosis in immunocompetent hosts in different states of the North and Northeast regions of Brazil (Trilles et al. 2008).

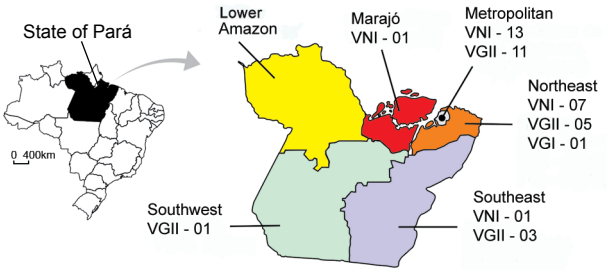
Most cases caused by the VGII and VNI types were from the metropolitan and Northeast mesoregions of Pará, both near the Hospital Universitário João de Barros Barreto. VGII and VNI types were also identified in more distant mesoregions of Pará such as Southeast, Southwest and Marajó. However, in the Lower Amazon mesoregion no case was identified, which may be due to the large distance that must be travelled (≥ 12 h by boat) to reach health services or the reference centre in the city of Belém. For the first time we demonstrate the occurrence and predominance of the molecular type VGII as an agent for cryptococcosis in normal individuals living in the state of Pará, including a significant number of paediatric cases. These paediatric cases came from dif-

TABLE

Patient data, clinical samples and results of *Cryptococcus* isolates on CGB test, serotyping, molecular typing by PCR-fingerprinting using the primer M13 and RFLP *URA5*, according to mesoregions of the state of Pará, Brazil

Patient	Gender	Age	HIV	Sample	LMM/WM number	CGB	Serotype	Mol. Type	Mesoregion - Belém
1	male	45	pos	CSF	1420 A/07207		UT	VNI	Metropolitan
				CSF	1420 B/07205	neg	UT	VNI	
2	male	20	pos	CSF	1421A/07206	neg	UT	VNI	Metropolitan
				CSF	1421B/07207	neg	UT	VNI	
3	male	43	pos	CSF	1423/07209	neg	A	VNI	Metropolitan
4	female	31	pos	CSF	1427/07213	neg	A	VNI	Metropolitan
5	male	27	pos	CSF	1433/07221	neg	A	VNI	Northeast
6	male	28	pos	CSF	1443/07233	neg	A	VNI	Northeast
7	male	58	pos	CSF	1452/07242	neg	A	VNI	Metropolitan
8	male	29	pos	CSF	1455/07245	neg	A	VNI	Northeast
9	male	33	pos	CSF	1457/07247	neg	A	VNI	Metropolitan
10	female	32	pos	CSF	1471/07249	neg	UT	VGII	Metropolitan
11	male	36	pos	CSF	1483 A/07253	neg	A	VNI	Northeast
				CSF	1483 B/07254	neg	A	VNI	Northeast
12	female	42	pos	CSF	1487/07259	neg	A	VNI	Metropolitan
13	male	52	pos	CSF	1463/ -	pos	UT	VGII	Metropolitan
14	male	34	pos	CSF	1468/ -	neg	A	VNI	Southeast
15	female	38	neg	CSF	1445/07235	neg	A	VNI	Northeast
16	male	15	neg	CSF	1422/07208	pos	B	VGII	Northeast
17	female	19	neg	CSF	1424/07210	neg	A	VNI	Metropolitan
18	female	15	neg	CSF	1425/07211	pos	B	VGII	Southwest
19	female	28	neg	CSF	1448/07238	neg	A	VNI	Metropolitan
20	female	35	neg	CSF	1450/07240	neg	A	VNI	Metropolitan
21	female	21	neg	CSF	1451/07241	pos	B	VGII	Metropolitan
22	female	60	neg	CSF	1426/07212	pos	B	VGII	Metropolitan
23	female	33	neg	CSF	1428/07214	pos	B	VGII	Metropolitan
				blood	1429/07215	pos	B	VGII	
24	female	30	neg	CSF	1432 A/07219	dub	B	VGII	Southeast
				CSF	1432 B/07220	dub	B	VGII	Southeast
25	male	65	neg	CSF	1434 A/07222	neg	UT	VNI	Metropolitan
				CSF	1434 B/07223	neg	UT	VNI	
26	male	42	neg	CSF	1453/07243	pos	B	VGII	Northeast
27	male	20	neg	CSF	1454/07244	pos	B	VGII	Metropolitan
28	male	32	neg	CSF	1458/07248	pos	B	VGII	Southeast
29	female	29	neg	CSF	1481/07252	neg	A	VNI	Marajó
30	female	24	neg	CSF	1486 A/07257	pos	B	VGII	Metropolitan
				CSF	1486 B/07258	pos	B	VGII	
31	female	42	neg	blood	1489/07269	neg	A	VNI	Northeast
				CSF	1492/07263	neg	A	VNI	
32	male	36	neg	CSF	1490/07261	neg	A	VNI	Metropolitan
33	male	17	neg	CSF	1491/07262	neg	A	VNI	Metropolitan
34	male	15	neg	CSF	1485 A/07255	pos	B	VGII	Metropolitan
				CSF	1485 B/07256	pos	B	VGII	
35	male	14	neg	CSF	1459/ -	pos	B	VGII	Metropolitan
36	female	9	neg	CSF	1430 A/07216	dub	UT	VGI	Northeast
				CSF	1430 B/07217	dub	UT	VGI	
				CSF	1436/07225	dub	UT	VGI	
37	male	5	neg	CSF	1431/07218	pos	B	VGII	Northeast
38	male	11	neg	CSF	1435/07224	pos	B	VGII	Metropolitan
39	male	8	neg	CSF	1441 A/07230	dub	UT	VGII	Northeast
				CSF	1441 B/07231	dub	UT	VGII	
40	male	7	neg	CSF	1444/07234	pos	B	VGII	Northeast
41	Female	11	neg	CSF	1449/07239	pos	B	VGII	Southeast
42	male	6	neg	CSF	1475 A/07250	dub	UT	VNI	Northeast
				CSF	1475 B/07251	dub	UT	VNI	
43	male	8	neg	CSF	1460/ -	pos	B	VGII	Metropolitan

CSF: cerebral spinal fluid; dub: dubious result; LMM: Pathogenic Fungi Collection, IPEC-Fiocruz; WM: Australian Medical Fungal Collection, Molecular Mycology Research Laboratory, Weastmead Hospital.



Distribution of 43 cases of cryptococcosis according genotype and mesoregions of the state of Pará, Brazil.

ferent mesoregions, suggesting that the environmental sources of VGII are widespread across the state. Additionally, one VGI case in a child from the Northeast mesoregion indicates that this molecular type may be also present in the local environment.

The mesoregions of Pará, with the exception of the metropolitan mesoregion, are mainly occupied by small communities and villages in the Amazon rainforest and share areas for cattle pastures and wood exploitation. The metropolitan mesoregion contains the areas of urbanization surrounding the city and harbour of Belém. At this port of the Amazon estuary, an extensive trade of wood and wood by-products, both for local use and for export, takes place. In Brazil, *C. gattii* and *C. neoformans* have been described in the decaying heartwood of a number of tree species, a possible primary ecological niche for both pathogens (Lazera et al. 2000). *C. gattii* was isolated from wood decay in a native jungle tree *(Guettarda acreana*) in Maracá, a river island without human activity in the Occidental Amazon rainforest, North Brazil (Fortes et al. 2001). This finding provides evidence that wild tropical forests may harbour *C. gattii*. The *C. gattii* isolated from the *Guettarda* tree was further identified as genotype AFLP6 (Trilles et al. 2003), which corresponds to the molecular type VGII.

C. gattii AFLP6/VGII seems to be well-adapted to the semiarid region in Northeastern Brazil, where it was isolated from the spleen of one armadillo, from wood decay in different trees and from human clinical specimens (Trilles et al. 2003). The genotype VGII is also the causative agent of the largest known outbreak infecting humans and animals and the first reported in a temperate area, Vancouver Island, Canada (Kidd et al. 2004). Considering the adaptive potential, expansive behaviour and pathogenicity of this molecular type, it is necessary to place accessible medical units in areas far from the city of Belém. These units will be able to provide specific medical information and laboratories for the early diagnosis, fungal isolation, identification and storage of the isolates. Additionally, these units can function as a registration system for the active surveillance of cryptococcosis in the state of Pará, Brazil.

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