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Original article

Molecular characterisation of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil

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Summary

As there are four major molecular types of *Cryptococcus neoformans* (VNI, VNII, VNIII and VNIV) and four molecular types of *Cryptococcus gattii* (VGI, VGII, VGIII and VGIV), it is important to identify the specific groups causing cryptococcosis in different geographical regions. Here, we investigated the molecular types of 57 cryptococcal isolates from patients in a tertiary care hospital in the state of Amazonas, Brazil, between 2006 and 2010. The isolates were characterised by PCR fingerprinting using the M13 minisatellite and confirmed by *URA*5-RFLP analysis, and the presence of specific genes from the mating type locus (MATα and MATa) of these species was analysed by PCR. Most of the patients were male (66.7%), between 16 and 30 years of age (51.7%), and HIV-positive (75.0%). Most isolates were collected from cerebrospinal fluid samples (71.7%). Most of the *C. neoformans* isolates (n = 40) were characterised as members of the VNI molecular group (n = 39), a unique isolate was characterised as VNII whereas all isolates of *C. gattii* (n = 17) were members of the VGII molecular group. With regard to mating types, 55 isolates were type 'α', and only two were type 'a'. This study revealed the prevalence of the VNI molecular group and provides the first reported observation of the VNII molecular group in the northern region of Brazil.

Key words: *Cryptococcus*, genotyping, PCR-fingerprinting, M13, *URA*5-RFLP, ITS1 RFLP analysis.

Introduction

Cryptococcosis is a systemic mycosis that affects the internal organs and skin and is caused by inhalation of infective forms of the pathogenic yeast species *Cryptococcus neoformans* and *Cryptococcus gattii*. This disease presents as subacute or chronic and affects both immunocompromised individuals (primarily AIDS patients) and immunocompetent individuals.[1,2]

Despite reproducing asexually, both *C. neoformans* and *C. gattii* have a complementary system with two mating types: 'a' and 'α'. For sexual reproduction to occur, there must be an encounter between two mating types. These mating types have being studied as they are involved in the virulence of these microorganisms.[1,2]

Cryptococcus neoformans has a worldwide geographical distribution, whereas *C. gattii* is most often found in tropical and subtropical regions. However, this pattern changed in 1998, when an outbreak of *C. gattii* infection was reported in the temperate climate of Vancouver Island, British Columbia, Canada, as characterised by PCR fingerprinting tools.[3–5]

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Molecular PCR fingerprinting techniques for typification are based on the amplification of PCR products from genomic regions that are conducive to study the phylogenetic relationships and permit distinctions at various taxonomic levels, e.g., species, variety or lineage.[6,7] Based on the studies by Meyer *et al.* [8], eight major molecular types have been defined by PCR fingerprinting with the M13 minisatellite and PCR combined with a restriction fragment length polymorphism (RFLP) analysis of the *URA*5 gene. The molecular types corresponding to *C. neoformans* are VNI, VNII, VNIII and VNIV, whereas *C. gattii* is subdivided into VGI, VGII, VGIII and VGIV.[9]

Studies on molecular typification improve fungal diagnoses, elucidate genetic diversity and corroborate global epidemiological studies. These studies are also important for associating different molecular characteristics with virulence and sensitivity to antifungals and therapeutics.[10–15]

The purpose of this study was to investigate the molecular types of the genus *Cryptococcus* isolated from outpatients at a tertiary healthcare unit in the state of Amazonas. Specifically, we aimed to (i) determine the molecular types by PCR fingerprinting using the M13 minisatellite and by *URA*5 RFLP analysis, (ii) analyse the presence of specific genes within the mating type locus (MATα and MATa); and (iii) characterise the patients according to gender, age, HIV infection status and type of clinical specimen from which the agents were isolated.

Materials and methods

Microorganisms

Fifty-seven isolates of *Cryptococcus* spp. were obtained from patients suffering from cryptococcosis who were admitted to The Fundação de Medicina Tropical, Dr. Hector Vieira Dourado between March 2006 and February 2010. Only the isolate obtained from the first sample processed for each patient was analysed and stored at (4 °C) in the FMT/HVD fungal collection. The microorganisms were reactivated on Sabouraud agar at 37 °C for 48 h. The microorganisms were subsequently seeded in Sabouraud broth at 30 °C for 48 h to obtain the fungal sample used for molecular characterisation. The standard strains 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) were used to be handled as reference during the characterisation. These strains were kindly supplied by the fungus collection from FIOCRUZ-Rio de Janeiro, Brazil.

Genomic DNA extraction

The QIAamp Blood and Tissue kit was used to extract DNA (Qiagen, Hilden, Germany)[12]. The genomic DNA concentration was determined by spectrophotometry (GeneQuant-pro RNA/DNA Calculator, GE Healthcare, Piscataway, NJ, USA) at a wavelength of 260 nm (absorbance unit corresponding to 50 μg ml^{-1}), and the purity was determined by the ratio between absorbances at 260 and 280 nm.

Characterisation of molecular types

PCR fingerprinting

This protocol employed a sequence specific for the M13 minisatellite (5′-GAGGGTGGCGGTTCT-3′), as described by Meyer *et al.* [9]. The amplification reaction was performed in a final volume of 25 μl. Each reaction contained 50 ng of DNA template, buffer solution [10 mM Tris–HCl (pH 8.3), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$], 0.2 mmol l^{-1} of each dNTP, 30 ng primer and 2.5 U of recombinant Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR was performed in a Verite 96 thermocycler (Applied Biosystems, Foster City, CA, USA). The reaction conditions consisted of 6 min of denaturation at 94 °C, 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 2 min of extension at 72 °C, and a final extension of 6 min at 72 °C. The amplification products were separated by electrophoresis on a 1.4% agarose gel for 6 h at 60 V.

URA5-RFLP analysis

This assay was performed as described by Meyer *et al.* [9]. Each reaction contained 50 ng of template DNA, buffer [10 mmol l^{-1} Tris–HCl (pH 8.3), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$], 0.2 mmol l^{-1} each dNTP, 50 ng of each *URA*5 primer (5′-ATGTCCTCCCAAGCCCTCGACTCCG-3′) and SJ01 primer (5′-TTAAGACCTCTGAACACCGTACTC-3′), and 1.5 U of recombinant Taq DNA polymerase (Invitrogen). The PCR was performed in a Verite 96 thermocycler (Applied Biosystems). The reaction consisted of 2 min of initial denaturation at 94 °C, 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 61 °C, 1 min of extension at 72 °C, and a final extension of 10 min at 72 °C. The size and purity of the PCR products were visualised by electrophoresis in a 1.5% agarose gel stained with SybrGreen and illuminated with ultraviolet light. Next, 8 μl of each PCR product was mixed with 1 ml buffer and digested with

Sau96I (10 U/μl) and *Hha*I (20 U/μl) for 3 h or overnight at 37 °C. The restriction fragments were analysed by electrophoresis on a 3% agarose gel for 5 h at 100 V.

Mating types

The mating types were determined by PCR[16], in a final volume of 25 μl. The α-type primers were Mat-αF (5′-CTTCACTGCCATCTTCACCA-3′) and Mat-αR (5′-GACACAAAGGGTCATGCCA-3′), and the a-type primers were Mat-aF (5′-CGCCTTCACTGCTACCTTCT-3′) and Mat-aR (5′-AACGCAAGAGTAAGTCGGGC-3′). Each PCR reaction contained 20 ng of template DNA, buffer solution [10 mmol l^{-1} Tris–HCl (pH 8.3), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$], 0.2 mmol l^{-1} of each dNTP, 20 ng of each primer and 1.5 U recombinant Taq DNA polymerase (Invitrogen). Amplification reactions were performed according to the modified procedure of Santos *et al.* [17]. Amplification products were analysed by electrophoresis on a 2% agarose gel for 3 h at 110 V.

Patient characteristics

Information on the patients' gender, age and HIV infection status as well as the type of clinical specimen from which the agents were isolated was obtained from the FMT/HVD mycology laboratory.

Results

The genus of the Cryptococcus isolates was determined based on their micromorphology characteristics in the nankin ink. The molecular methodologies used in this work allowed to demonstrate that of the 57 isolates, 40 (70.2%) were characterised as *C. neoformans*, and 17 (29.8%) were *C. gattii*.

The PCR fingerprinting (Fig. 1) and *URA*5 PCR RFLP (Fig. 2) analyses revealed that the molecular types present were VNI (39/57, 68,4%), VNII (1/57; 1.8%) and VGII (17/57; 29.8%). The data obtained from the two methodologies were in complete agreement.

As shown in Table 1, further examination of the available patient information revealed a prevalence of males in the study (38/57; 66.7%). Moreover, close to half of the patients were between 16 and 30 years of age (29/57; 50.9%) and HIV-positive (44/57; 77.2%). The isolates were primarily derived from cerebrospinal fluid (CSF) (42/57; 73.7%). With regard to age, we observed that *C. gattii* (VGII) infections were seen in all

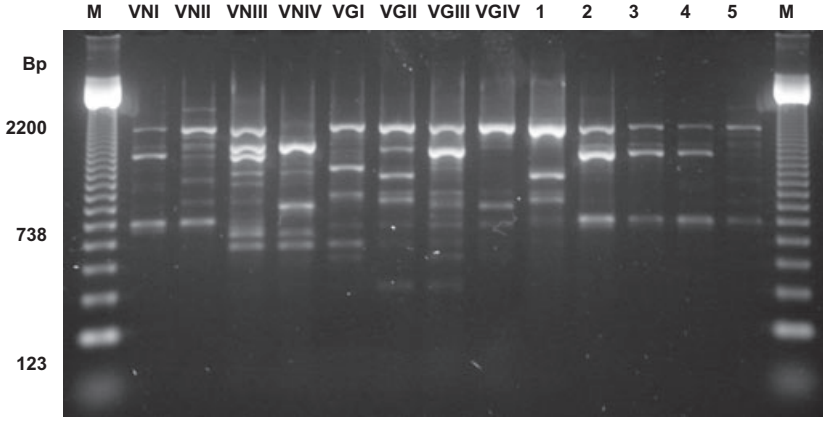

Figure 1 PCR fingerprinting profiles obtained with the M13 minisatellite primer in reference *Cryptococcus* spp. strains (lanes 2–9) and patient isolates (samples marked 1, 2, 3, 4 and 5). M, DNA size marker.

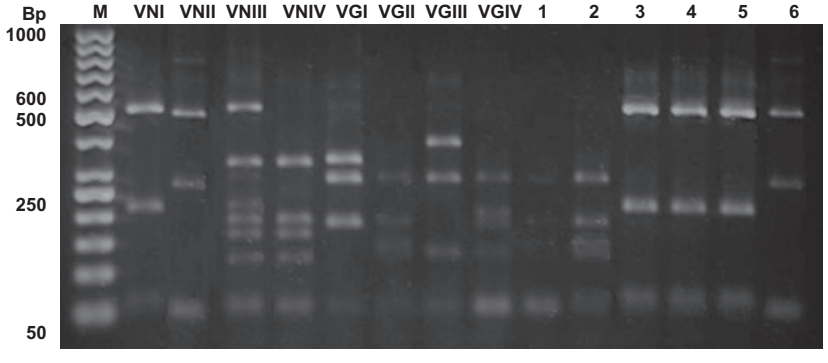

Figure 2 *URA*5-RFLP profiles obtained after digestion with the restriction enzymes *HhaI* and *Sau96I* in reference *Cryptococcus* spp. strains (lanes 2–9) and patient isolates (samples marked 1, 2, 3, 4, 5 and 6). M, DNA size marker.

Table 1 Molecular groups of Cryptococcus isolates and patient characteristics.

Species *n* (%)	Molecular group (*n*)	Gender *n* (%)		Age *n* (%)					Biological sample *n* (%)				HIV infection *n* (%)	
		M	F	0–15	16–30	31–45	46–60	>60	CSF	Hemoculture	Sputum	Other[1]	Yes	No
C. neoformans 40 (70.2)	VNI (39)	25 (43.9)	14 (24.6)	–	23 (40.4)	13 (22.8)	3 (5.3)	–	31 (54.4)	3 (5.3)	1 (1.8)	4 (7.0)	36 (63.2)	3 (5.3)
	VNII (1)	1 (1.8)	–	–	–	1 (1.8)	–	–	–	1 (1.8)	–	–	1 (1.8)	–
C. gattii 17 (29.8)	VGII (17)	12 (21.1)	5 (8.8)	4 (7.0)	6 (10.5)	3 (5,3)	2 (3.5)	2 (3.5)	11 (19.3)	–	1 (1.8)	5 (8.8)	7 (12.3)	10 (17.5)
Total *n* (%)		38 (66.7)	19 (33.3)	4 (7.0)	29 (50.9)	17 (29.8)	5 (8.8)	2 (3.5)	42 (73.7)	4 (7.0)	2 (3.5)	9 (15.8)	44 (77.2)	13 (22.8)

[1]Samples included bronchial lavage, ascitic fluid, bone marrow and urine.

age groups and that four (7%) cases of the disease occurred in children aged 0–15 years. The VNII isolate was obtained from a blood culture of a 36-year-old male who was HIV-positive.

Investigating the mating types of the cryptococcal isolates, it was observed that 55 were type 'α' isolates and two were type 'a' samples. The type 'a' isolates belonged to the molecular type VNI.

Discussion

Cryptococcus neoformans was the predominant fungal species isolated from this group of Brazilian patients (70, 2%). This agent causes disease almost mainly in immunosuppressed patients. Indeed, most isolates examined in this study were obtained from patients with AIDS. These findings are in agreement with other studies demonstrating that this species is responsible for up to 82.3% of cases of infection with this genus.[9,18] A study of Latin American isolates from nine countries, including Brazil, revealed that a majority of the 340 strains of *C. neoformans* belonged to the VNI group. In the 66 Brazilian strains, three molecular types were found, with VNI being predominant at 82.3%, followed by VGII (13.6%) and VNII (3.0%). However, this study did not indicate the states or regions from which the isolates were obtained.[9] In an important survey of cryptococcosis in southern Brazil, Casali *et al.* [19] showed that 105 clinical isolates and 19 environmental isolates were *C. neoformans*, with a predominance of the VNI molecular group. In 2008, Trilles *et al.* [18] performed a study to determine the distribution of molecular types among eleven states in Brazil. These authors analysed 443 isolates, 320 of which were *C. neoformans* and 123 of which were *C. gattii*. Twelve of these isolates were obtained in the state of Amazonas during the 1990s and during the characterisation it was demonstrated that they belonged to the VNI and VGII molecular types.

In our study, one isolate (1.8%) was characterised as VNII. This finding is in agreement with the literature, which has shown that this is the third most prevalent molecular group, with a frequency of 3–5% in analyses performed in Brazil.[18] However, no isolate from the state of Amazonas analysed in previous studies was characterised as belonging to this molecular group.[18,20] Thus, this is the first report of the existence of *C. neoformans* of the VNII molecular group in northern Brazil.

Among the 17 strains of *C. gattii* examined in our study, ten were obtained from patients who showed no sign of being immunocompromised and seven where obtained from patients with AIDS. *C. gattii* is considered

a primary pathogen; however, recent studies have shown that this pathogen also infects immunocompromised patients.[21,22] All *C. gattii* isolates found in our study belonged to the VGII molecular group, consistent with recent studies demonstrating that this group is the main cause of the disease in immunocompetent patients in different states of north and northeastern Brazil.[18,20,23]

The α mating type is generally more prevalent than the a type in clinical samples, as observed in the present study. The higher prevalence of the α mating type is because of the selective advantages of longer survival in the environment and greater virulence.[16,24,25]

The present study also found that male patients, patients aged 16–30 years, and HIV-positive patients are the groups most commonly affected by cryptococcosis in the state of Amazonas in Brazil. The literature also notes that men and individuals 16–30 years of age are most frequently affected by this infection, probably because of the AIDS epidemiology.[26] More recently, it was suggested that female hormones may offer women some protection against cryptococcosis.[25]

Our observation of *C. gattii* as a causative agent of meningitis in HIV-positive children 0–15 years old is in accordance with the literature.[17,27,28] A study conducted in the state of Amazonas demonstrated that children with cryptococcosis accounted for a significant fraction of the reported cases of meningitis between 1988 and 1998 (33%, $n = 75$).[27] Also in the northern region, in the Para state, over a period of 7 years, 24% ($n = 78$) of patients hospitalised with cryptococcosis were children.[28] This high frequency of cryptococcal meningitis was also observed between 2003 and 2007 (8/43, 18.6%) in the same state.[17] In the northeastern Piauí and Maranhão states, 21% ($n = 257$) of meningitis cases were children with cryptococcosis.[29] Further study of the aetiopathogenesis of cryptococcosis in humans or, more specifically, in children, as well as the molecular profiles of these patients, should be performed because data concerning childhood infections are of great interest.

Most of the fungal isolates used in this study were retrieved from CSF samples. This result can be explained by the fact that the majority of patients with the cryptococcosis present meningitis[1,2]. Our data therefore align with literature reporting a high prevalence of fungal isolation from the CSF. In a recent study in the Amazonas state, a large proportion of HIV infected individuals developed cryptococcal meningitis (10%).[20] This clinical presentation is one of the leading causes of morbidity and mortality in AIDS patients. In many individuals, this mycosis is the first indication that the HIV infection has evolved into the symptoms of AIDS.[30]

In conclusion, cryptococcosis in the Amazonas state continues to be prevalent in HIV patients, with *C. neoformans* of the VNI molecular group as the main aetiological agent. However, we also detected the VNII molecular group of this species, which has never been reported in the northern region of Brazil.

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