

Original Articles

Genotype and mating type distribution within clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from patients with cryptococcal meningitis in Uberaba, Minas Gerais, Brazil

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We molecularly characterized 81 cryptococcal isolates recovered from cerebrospinal fluid samples of 77 patients diagnosed between 1998 and 2007 as having cryptococcal meningitis in Uberaba Minas Gerais, Brazil. Fifty-seven (74%) were male with a mean age 35.6 years. Seventy-two (88.9%) of the isolates were from 68 AIDS patients and cryptococcosis was the first AIDS-defining condition in 38 (55.9%) patients. Cryptococcosis and AIDS were simultaneously diagnosed in 25 (65.8%) of these 38 patients. Genotypes were characterized through the use of *URA5* restriction fragment length polymorphisms analysis, the genetic variability was determined using PCR-fingerprinting with the minisatellite-specific primer M13, and the mating type and serotypes were established by PCR. Seventy-six of the 81 isolates were *Cryptococcus neoformans* (93.8%), while the remaining five were *C. gattii* (6.1%), but all were mating type α . *C. neoformans* isolates were genotype VNI (serotype A), while *C. gattii* isolates were VGII. Four of the latter isolates were identical, but only two were from AIDS patients. Six of the nine isolates from non-AIDS patients were VNI. PCR fingerprints of the isolates from two of the three AIDS patients with clinical relapse were 100% identical. The predominance of VNI and mating type α is in accordance with data from other parts of the world. The occurrence of VGII in Minas Gerais indicates a geographical expansion within Brazil.

Keywords *Cryptococcus neoformans*, *Cryptococcus gattii*, mating type, *URA5*-RFLP, PCR-fingerprinting

Introduction

Recently, *Cryptococcus neoformans* and *Cryptococcus gattii* were recognized as two different species based on phenetic, as well as the biologic species concepts, beyond molecular

differences in their genetic structure [1,2]. *Cryptococcus neoformans* has been found to be composed of two varieties and three serotypes based on molecular analysis, genome sequence and serological tests, i.e., *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and the hybrid (serotype AD) [1,3,4]. *C. gattii* contains two serotypes, B and C, although the boundaries between serotypes and molecular types are not distinguishable [1,3,5,6].

The etiologic agents of cryptococcosis differ in their virulence, geographical distribution, pathogenicity, clinical picture and the therapeutic outcomes of infections [1–3].

Received 20 April 2009; Received in final revised form 17 September 2009; Accepted 22 September 2009

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Serotype A is recovered worldwide and accounts for over 90% of all cryptococcal infections and more than 99% of cryptococcosis cases in AIDS patients [7,8]. Mortality is up to 30% in those regions where the highly active antiretroviral therapy (HAART) is not yet available or patients only poorly adhered to prescribed therapy [9].

Classically, *C. gattii* affects healthy hosts living in tropical and subtropical rural areas and it is occasionally isolated from AIDS patients. However, in some African regions it may be the etiologic agent in up to 10% of the cases [10,11]. However, its geographical boundaries have expanded with reports of the recent outbreak of meningoencephalitis among residents, visitors, domestic and wild animals in Vancouver Island, British Columbia, Canada [12]. In addition, *C. gattii* was also isolated in the Pacific Northwest region of the United States and in Europe [12–14].

The mating type has been identified as a potential virulence factor and can play an important role in the epidemiology and evolution of this microorganism [15,16]. *C. neoformans* and *C. gattii* have a known sexual phase involving two mating types, *MATa* and *MAT α* [17,18]. *MAT α* isolates are the predominant mating type recovered from environmental and clinical sources. They are more virulent in animal models and show greater tropism to the central nervous system (CNS) than the *MATa* strains [19–21].

The *Cryptococcus* species complex has been divided into eight major molecular types by PCR-fingerprinting using single primers specific to microsatellite [(GACA)₄ and (GTC)₅], or minisatellite (M13) repeats, amplified fragment length polymorphism analysis (AFLP) and PCR-RFLP analysis (*URA5*, *PLB1* genes). Results indicate that VNI and VNII genotypes correspond to serotype A, VNIII is related to serotype AD and VNIV to serotype D of *C. neoformans*. However, VGI–VGIV contain isolates of both serotypes (B and C) of *C. gattii* [4,22–24].

The VNI genotype is distributed worldwide and recovered mainly from AIDS patients, whereas VGI is associated with infection in immunocompetent hosts and is the most prevalent genotype in Asian countries [25,26]. The VGII genotype is distributed in Oceania, and North and South American countries [12,14,27,28]. Most clinical and environmental isolates from the South, Southeast and Central-West Brazilian regions are VNI *MAT α* , whereas the VGII *MAT α* predominates in the North and Northeastern regions of Brazil, especially in immunocompetent young adults and children [29–33].

The aim of this study was to identify the genotypes, the genetic variability, serotypes and mating types by molecular analyses of clinical isolates of *Cryptococcus* spp. obtained from patients with cryptococcal meningitis diagnosed at a teaching hospital located in the Southeast region of Brazil.

Materials and methods

Patients and samples

A total of 81 isolates were recovered from cerebrospinal fluid (CSF) samples of 77 patients with cryptococcal meningitis diagnosed from 1998 to 2007 at the teaching hospital in Uberaba, Minas Gerais State. The medical records of these patients were reviewed for the clinical data. The study was approved by the Ethical Board of the Triângulo Mineiro Federal University (protocol number 678).

Isolation, identification and maintenance of the isolates

The identification of suspected yeast isolates included standardized morphological, biochemical and physiological tests, such as L-Canavanine–Glycine–Bromothymol blue media (CGB), India ink, pigment production on Niger Seed Agar medium (NSA), nitrate and carbon assimilation, positive urease reaction and thermotolerance at 37°C [34]. Each *Cryptococcus* isolate was sub-cultured on NSA medium and a single colony was selected for molecular analysis. The isolates were preserved in both glycerol stocks at –20°C and lyophilized with Skim Milk (DIFCO, Laboratories, Detroit, MI, USA).

DNA extraction

Genomic DNA was obtained from cultured cells as previously described by Ferrer *et al.*, with some modifications [35]. Yeasts were grown on plates containing YEPD at 37°C for 2–3 days, after which approximately 150 mg of the yeast cell pellet were put in a microtube and frozen at –20°C overnight. Five hundred μ L of cell lysis solution (1.5% NaCl, 0.5% sodium dodecyl sulfate, 0.25M EDTA, pH 8.0 and Tris-HCl 10mM, pH 7.5) and 5 μ L of 2-mercapto-ethanol (Sigma Aldrich, USA) was then added to the microtube. The tube was then incubated and vortexed for 5 min at room temperature prior to its incubation in a dry bath at 65°C for 1 h, with occasional shaking. The lysate was centrifuged for 15 min at 14,100 g, and the aqueous phase was transferred to a new tube. Then a mixture of 500 μ L phenol-chloroform-isoamyl alcohol (v:v:v 25:24:1) was added, mixed thoroughly for 4 min to obtain a homogenous suspension, and then centrifuged for 15 min at 14,100 g. To precipitate the DNA, cold isopropanol (1:1) was added, the tube gently shaken and incubated at –20°C overnight. The DNA pellet was then washed with 70% ethanol, air dried and suspended in 500 μ L TE (10 mM Tris-HCl, pH 7.5, 0.5 M EDTA, pH 8.0) containing 50 μ g/mL RNase A (Invitrogen, USA), incubated at 37°C for 40 min and stored at –20°C. The integrity of the extracted DNA was detected on a 1% agarose gel and visualized under UV light. The DNA concentration was determined spectrophotometrically at 260/280 nm.

Genotype determination by *URA5-RFLP*

Amplification reactions of the *URA5* gene were performed in a final volume of 50 μ L as described previously [23]. Each reaction contained 50 ng of DNA, 1 X PCR buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 2mM MgCl₂, 1.5 U of Taq DNA polymerase and 50 ng of each primer *URA5* (5'-ATGTCCTCCCAAGCCCTCGACTCCG-3') and *SJ01* (5'-TTAAGACCTCTGAACACCGTACTC-3') (Invitrogen, Brazil). The PCR consisted of an initial denaturation at 95°C for 4 min, followed by 34 cycles amplification: 45 s of denaturation at 94°C, 1 min of annealing at 57°C, 1 min of extension at 72°C. A final extension step was applied at 72°C for 10 min. A volume of 30 μ L of the PCR amplicons was double digested with *Sau96I* (10 U/ μ L) and *HhaI* (20 U/ μ L) (New England Biolabs, USA) and then incubated in a dry bath at 37°C for 3 h. The restricted fragments were separated by electrophoresis on 3% agarose gels at 100 V for 5 h. *URA5-RFLP* patterns were assigned visually by comparison with patterns obtained from standard strains representing eight molecular types [23]. Bands were included in the analysis regardless of their intensity.

PCR fingerprinting

The minisatellite-specific core sequence of the wild-type phage M13 (5'-GAGGGTGGCGGTTCT-3') was used as single primer in the PCR-fingerprinting in a slightly modified method which was originally described by Meyer *et al.* [22]. Briefly, the amplification reaction was conducted in a volume of 50 μ L containing 100 ng of DNA, 1 x PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.0 mM MgCl₂, 60 ng primer (Invitrogen, Brazil) and 2.5 U of Taq DNA polymerase (Invitrogen, Brazil). The PCR consisted of an initial denaturation at 94°C for 10 min, followed by 36 cycles amplification: 1 min of denaturation at 94°C, 1 min of annealing at 50°C, 1 min of extension at 72°C. A final extension step was applied at 72°C for 10 min. Amplification products were separated by electrophoresis in 1.5% agarose gels in 1 x Tris-Acetate EDTA (TAE) buffer at 90 V for 4.5 h and stained with ethidium bromide at 0.5 μ g/mL for 30 min and visualized under UV light. The molecular types (VNI-VNIV and VGI-VGIV) were assigned by comparison to the reference strains of the eight major molecular types [22,23]. The reference strains were used as internal controls to confirm reproducibility.

The PCR fingerprinting profiles were analyzed on the basis of the presence or absence of clear and defined bands in the digitalized gel images were used to build the dendrograms. Data were processed with the program Gel

Compar II. The similarity matrix and clustering dendrograms were calculated using the Jaccard coefficient and Unweighted Pair-Group Method Arithmetic averages (UPGA) algorithm, respectively.

Determination of mating type by PCR

Two specific PCR primers pairs for mating type α and **a** were used according to Chaturvedi *et al.* [36]. The α -mating type specific primers were *MAT α F* (5'-CTTCACTGCCA-TCTTACCA-3') and *MAT α R* (5'-GACACAAAGGGT-CATGCCA-3'). The **a**-mating type specific primers were *MAT**a**F* (5'-CGCCTTCACTGCTACCTTCT-3') and *MAT**a**R* (5'-AACGCAAGAGTAAGTCGGGC-3'). Amplification reactions were performed in a final volume of 25 μ L. The master mix contained 25 ng of genomic DNA, 2 mM MgCl₂; 15 ng of the forward and reverse primers, PCR buffer 1X (10 mM Tris-HCl, pH 8.0; 50 mM KCl); 0,25 μ L dNTP 20 mM; 1.5 U of Taq DNA polymerase (Invitrogen, Brazil). PCR reactions were performed in a MJ Research PTC 100 thermocycler (MJ Research, Watertown, MA, USA). Initial denaturation was done at 95°C for 4 min, followed by 34 cycles of amplification: 1 min of denaturation at 94°C, 1 min of annealing at 63°C or 60°C for (*MAT α* or *MAT**a***, respectively), 1 min of extension at 72°C and 10 min of final extension at 72°C. The PCR amplicons were loaded on 2% agarose gels in TAE buffer at 80 V for 90 min, and then stained in a solution of ethidium bromide at 0.5 μ g/mL and visualized under UV light. The presence of 101 and 117 bp fragments was considered positive for the mating type α and **a**, respectively.

Determination of serotype by PCR

In order to establish the concordance between VNI genotype and the serotype A, PCR studies were performed using serotype and mating type-specific primers based on the *STE20 α A* gene sequence [37]. Amplification reactions were performed in a final volume of 50 μ L, with each reaction containing 40 ng of DNA, 1 X PCR buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 2mM MgCl₂, 1.5 U of Taq DNA polymerase and 50 ng of each primer *JOHE7264* (5'-AGCTGATGCTGTGGATT-GAATAC-3') and *JOHE7265* (5'-GTTCAATTAATCTCAC-TACCTGTAG-3') (Invitrogen, Brazil). The PCR consisted of an initial denaturation at 94°C for 10 min, followed by 39 cycles amplification: 1 min of denaturation at 94°C, 1 min of annealing at 48°C, 1 min of extension at 72°C. A final extension step was applied at 72°C for 10 min. The PCR amplicons were electrophoresed on 2% agarose gels in TAE buffer at 80 V for 90 min and then stained in a solution of ethidium bromide at 0.5 μ g/mL and visualized under UV light. The presence of a 1200 bp fragments was considered positive for the serotype A and mating type α .

Reference strains

The standard strains (ATCC [American Type Culture Collection, Manassas, VA, USA] 90112 serotype A *MAT* α and ATCC 28958 *MAT* α) were obtained from Celular and Molecular Department of São Paulo University, Ribeirão Preto, Brazil. The reference strains were obtained from the Cryptococcal Culture Collection at Mycology Laboratory, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil and represented each of the eight major molecular types as follows; WM 148 = LMM 794 (serotype A, VNI/AFLP1), WM 626 = LMM 795 (serotype A, VNII/AFLP1A), WM 628 = LMM 796 (serotype AD, VNIII/AFLP2), WM 629 = LMM 797 (serotype D, VNIV/AFLP3), WM 179 = LMM 798 (serotype B, VGI/AFLP4), WM 178 = LMM 799 (serotype B, VGII/AFLP6), WM 175 = LMM 800 (serotype B, VGIII/AFLP5), and WM 779 = LMM 801 (serotype C, VGIV/AFLP7) as previously reported by Meyer *et al.* [23] and Trilles *et al.* [38].

Results

Clinical isolates

A total of 77 patients with cryptococcal meningitis were diagnosed over the last ten years at the teaching hospital

in Uberaba, Minas Gerais, Brazil. Fifty-seven (74%) were male, with a mean age of 35.6 (15–62 years) years. Sixty-eight (88.3%) were AIDS patients and of the remaining nine, one was a renal transplant recipient, two presented with systemic lupus erythematosus and six had no underlying immunosuppression. Cryptococcosis was the first AIDS-defining disease in 38 (55.9%) cases, while in 25 (65.8%) of these 38 cases, the two diseases were simultaneously diagnoses at admission.

Molecular characterization of the isolates

The *URA5*-RFLP analysis of 81 isolates identified 76 (93.83%) as *C. neoformans*, VNI genotype, whereas five (6.17%) isolates were *C. gattii*, VGII genotype. All isolates were mating type α (Figs. 1 and 2, Table 1). The VNI isolates were divided into 10 subtypes by PCR-fingerprinting and just one of these subtypes included 43 (56.5%) isolates, with 100% of similarity. Six out of nine isolates obtained from non-AIDS patients belonged to the VNI genotype. Of these, four presented similar profiles (isolates L403, L646, L473 and L453) whereas isolates L534 and L140 showed only 82.7% similarity among them (Fig. 3). Four of the five VGII isolates had a high similarity by PCR fingerprinting but only two of these had been recovered

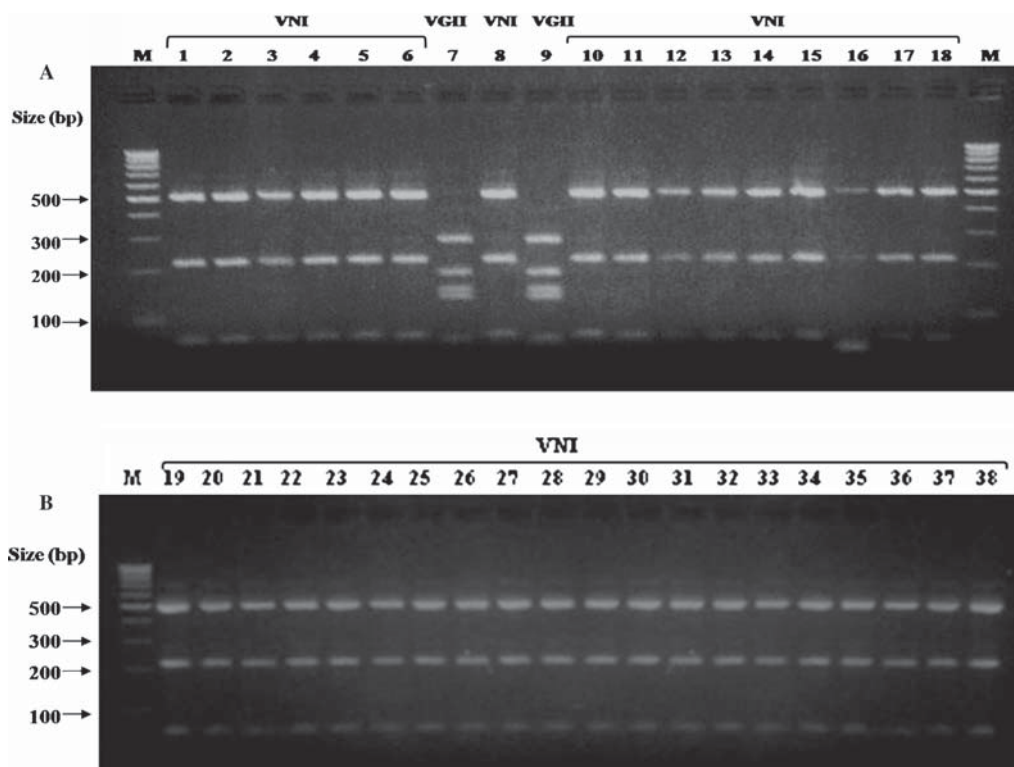


Fig. 1 Examples of the *URA5* RFLP analysis obtained after double digestion with the enzymes *Sau96I* (10 U/ μ L) and *HhaI* (20 U/ μ L) representing the VNI (*Cryptococcus neoformans* var. *grubii*) and VGII (*Cryptococcus gattii*) genotypes. A and B Lane 1–38 clinical isolates M- 100 bp DNA ladder (Gibco-BRL, USA).



Fig. 2 Examples of the *MATα* locus amplification obtained with the primers *MATα1* and *MATα2* of the *MFα* pheromone gene. C = negative control (ATCC 28958); Lane 1 = positive control (ATCC 90112); Lanes 2–19 = clinical isolates. M = 100 pb DNA ladder (Gibco-BRL, USA).

from AIDS patients (isolates L261 and L747) (Fig. 4B). The one remaining VGII isolate (L667) showed 93.3% similarity by PCR fingerprinting profile to the other four. Because all *C. neoformans* strains were genotype VNI, mating type α , a PCR test was performed using serotype and mating type specific primers based on the *STE20* gene sequences and it amplified a band of 1200 bp specific for the serotype A α allele in all isolates (Fig. 5).

Three of the 72 AIDS patients presented with a cryptococcal meningitis relapse after two or more years from the first infection event. The new isolates were VNI, mating type α . Isolates from two of these patients showed 100% similarity in their PCR fingerprinting patterns, patient 1 (L322, L332 and L342) and patient 2 (L130 and L140). However, isolates of patient 3 (L767, L787) presented only a similarity of 83.3% (Figs. 3 and 4).

Discussion

Cryptococcal meningitis has been considered as a defining condition of AIDS since the first years of the AIDS era and represents the most common fungal infection of the CNS and the third most frequent neurological related event in these patients [39,40]. In this study, we analyzed

76 different clinical isolates of *C. neoformans* and five *C. gattii* recovered from 77 patients.

Of the two major genotypes identified, VNI was the most prevalent representing (93.82%) 76 strains obtained from 66 AIDS patients and from six non-AIDS patients. Similar results have been observed in other studies with clinical isolates from around the world, e.g., Malaysia 42 (95.5%), Guatemala 15 (93.3%), Peru 13 (93.3%), China 115 (89.5%) and India 51 (89.5%) [23,25,26,41]. In Brazil, 95.7%, 82.9% and 82.9% of isolates from the states of São Paulo, Rio Grande do Sul and Rio de Janeiro, respectively, were this genotype [29–31]. Recently, another survey, which included *Cryptococcus* isolates from several regions of Brazil showed a prevalence of 64% of the VNI genotype, while in the Southern region the prevalence was 74.5% [38]. This figure differs from the 93.8% rate found in the present study carried out in the same area.

Most of the cryptococcal infections in AIDS patients from other parts of the world are caused by the VNI genotype (serotype A). However, individuals with or without other underlying immunosuppression may be infected by this genotype as evidenced in six (7.8%) subjects in this study. Previous reports have already confirmed this finding [23,25,38].

Table 1 Summary of the molecular characterization of the 81 strains of *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from the cerebrospinal fluid of patients with cryptococcal meningitis in Uberaba, Minas Gerais, Brazil

Year of isolation	No. of strains	Canavanine-glycine-bromothymol blue (CGB)		Mating type <i>MATα</i>	Genotype	
		<i>C. neoformans</i>	<i>C. gattii</i>		VNI	VGII
1999	5	5	-	5	5	-
2000	9	9	-	9	9	-
2001	12	9	3	12	9	3
2002	12	12	-	12	12	-
2003	10	10	-	10	10	-
2004	8	8	-	8	8	-
2005	5	5	-	5	5	-
2006	5	4	1	5	4	1
2007	15	14	1	16	15	1
Total	81	76	5	81	76	5

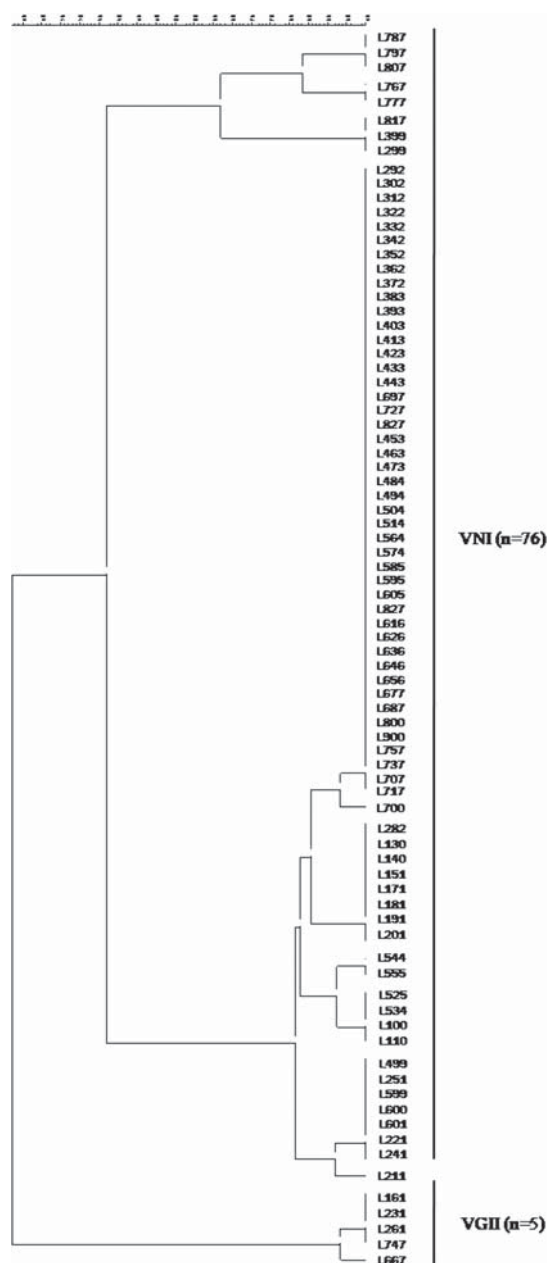


Fig. 3 Phenogram showing the relationships of the 81 clinical isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* generated from the PCR-fingerprinting profiles obtained with the primer M13 (GelCompar II).

The VNI genotype was also identified in 84 (70%) *C. neoformans* strains isolated from apparently healthy individuals in China. However, multilocus sequence typing (MLST) demonstrated that these isolates belonged to a cluster phylogenetically divergent from the VNI reference strain [42].

The prevalence of the VGII genotype is similar in Colombia (99.2%), in the Northern and Western regions of Australia (71%), Northwestern United States (80%) and Canada (95%) [14,28,43,44]. In the last few years, the

largest known outbreak of cryptococcal meningitis caused by VGII was described on Vancouver Island, British Columbia, Canada among residents, visitors, and domestic and wild animals [12]. Autochthonous infection by this genotype was also recently detected in other areas in the Pacific Northwest of the USA in human and animals, but no links of these cases with Vancouver Island or other endemic areas has yet been determined [14].

Five out of the 81 clinical isolates in this study were *C. gattii*, VGII genotype, as shown by two molecular methods and the latter permitted us to separate them into two different clusters. However, it was not possible to establish the origin and the patient's migratory profile. Previous reports from Southern Brazil showed a prevalence of 48% of VGII among 50 clinical isolates but most of them had been recovered from patients who had migrated from the Northeast where this genotype represents 89% of all *C. gattii* infections [38]. Similar figures were also found in the north along the Amazon basin [33].

Cryptococcal meningitis by *C. gattii* was diagnosed in 2 (3.3 %) of the 66 AIDS patients investigated in this study. This finding confirms other reports from South, Southeast and the Middle regions of Brazil where most of the AIDS patients reside and a low prevalence of HIV/*C. gattii* co-infection has been observed [45,46]. In contrast, rates as high as 8–10% of HIV/*C. gattii* co-infections were reported from Botswana, Malawi, Southern California and Northern Brazil [11,47–49].

All isolates studied here were mating type α . This feature is in accordance with several studies of clinical isolates reported from Canada, Brazil, the United States and China [8,12,31,42]. Other surveys of a great number of clinical and environmental isolates in South America have found 1.9% and 5% of isolates were mating type **a** which confirms the low prevalence of this mating type for both species compared to the mating type α [29,38]. Of special note is an analysis of 119 VGII isolates from Colombia, which showed a prevalence rate of 96.6% of mating type **a** [28].

Comparison of isolates from three AIDS patients who relapsed two years or more after the first episode found that two of them had VNI genotypes with 100% similarity of their PCR fingerprinting patterns. This suggests that the two clinical episodes in each patient might have been caused by the same strain whose origin could be either endogenous reactivation or a re-infection from the same infection source. On the other hand, the isolates of the third patient showed only a 83.3% similarity, belonging to two different clusters, which suggests that another *Cryptococcus* strain may be the cause of the clinical relapse. However, several studies have shown that endogenous reactivation of cryptococcal infections is more frequently related to relapse events [50,51].

The predominance of the VNI genotype and mating type α in isolates from AIDS patients in this study is in accordance with reports from other parts of the world

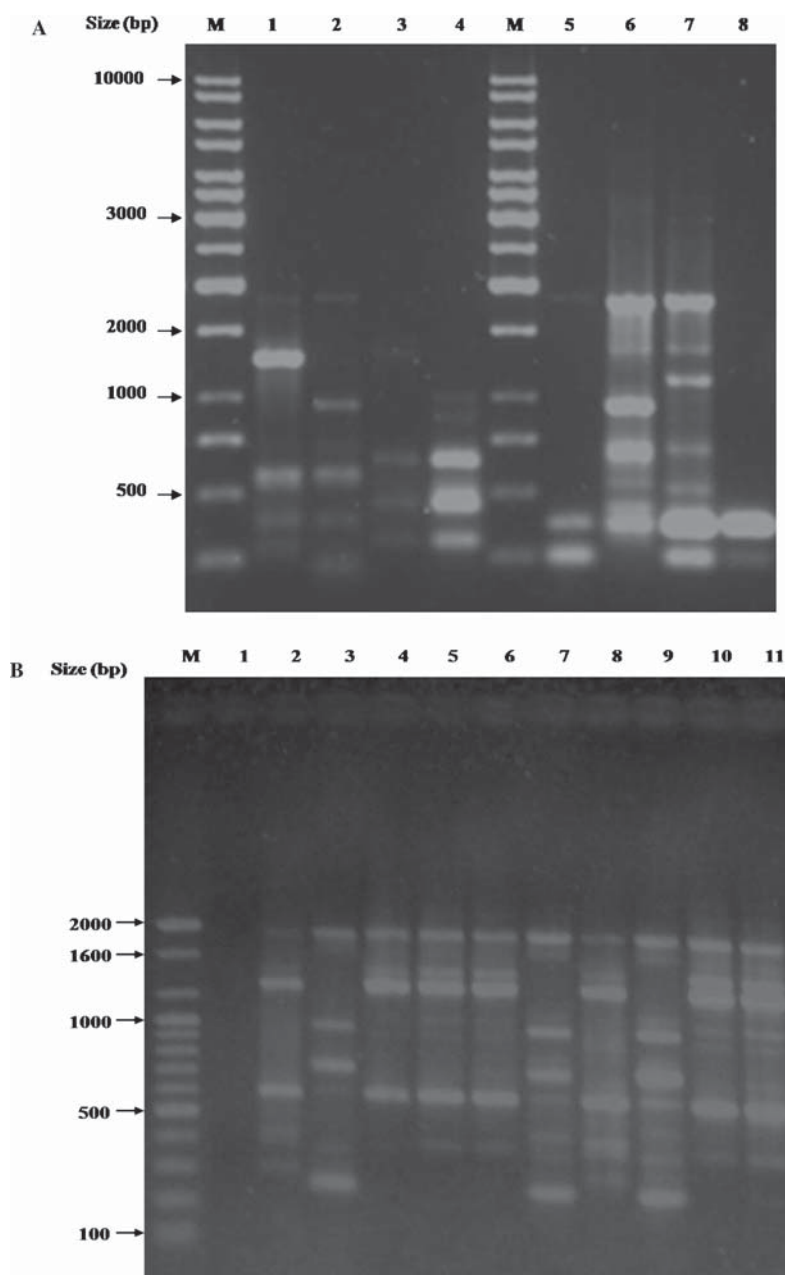


Fig. 4 (A) PCR-fingerprinting analysis of *Cryptococcus neoformans* and *Cryptococcus gattii* reference strains. M: 1Kb DNA marker (Fermentas), Lane 1: WM 148 (VNI), lane 2: WM 626 (VNII), lane 3: WM 628 (VNIII), lane 4: WM 629 (VNIV), lane 5: WM 179 (VGI), lane 6: WM 178 (VGII), lane 7: WM 175 (VGIII), lane 8: WM 779 (VGIV). (B) Representative PCR-fingerprinting profiles obtained with single primer M13 obtained from a selection of clinical isolates: M: 100 bp DNA ladder (Gibco-BRL, USA), Lane 1: negative control; lane 2: L767 (VNI); lane 3: L 667 (VGII); lane 4: L322 (VNI); lane 5: L332 (VNI); lane 6: L342 (VNI); lane 7: L261 (VGII); lane 8: L787 (VNI); lane 9: L747 (VGII); lane 10: L130 (VNI); lane 11: L140 (VNI).

[8,12,31,42]. In addition the occurrence of five VGII cases in Southern Brazil emphasizes the relevance to molecular characterization of clinical and environmental *Cryptococcus* isolates in order to understand the geographical expansion of this genotype and the relation of strains with the specific sub-genotypes associated with the Vancouver Island outbreak.

Acknowledgments

This research was supported by Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG) grant # CDS-1881/6. We thank Miss Angela Azor, Prof. Reginaldo dos Santos Pedroso and Mr Celso Tadeu Barbosa dos Santos for their technical support, Mr Henrique

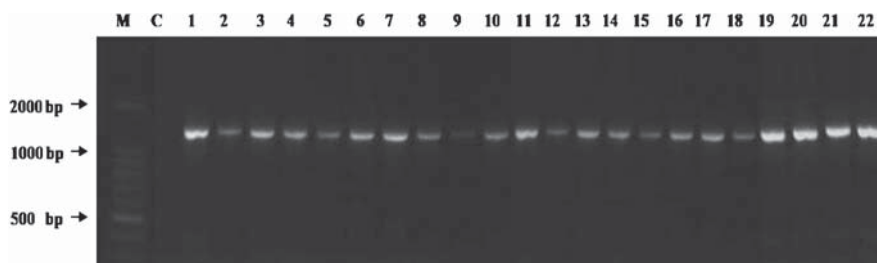


Fig. 5 Examples of the serotype A *MATα* amplification obtained with the primers *JOHE7264* and *JOHE7265* of *STE20Aα* gene, representing strains of the serotype A, *MATα*, VNI genotype. C = negative control (ATCC 28958); Lane 1 = positive control (ATCC 90112); Lanes 2–22 = clinical isolates. M = 100 bp (Gibco-BRL, USA).

Borges Kappel and Mrs Daniela Stefani Marques for their help with the generation of the dendogram and A/Prof. Wieland Meyer for the critical review of this manuscript.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 11 November 2009.