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

DELIO JOSÉ MORA*, ANDRÉ LUIZ PEDROSA*, VIRMONDES RODRIGUES*, CLAUDIA MARIA LEITE MAFFEI†, LUCIANA TRILLES‡, MÁRCIA DOS SANTOS LAZÉRA‡ & MARIO LEÓN SILVA-VERGARA*

*Disciplina de Doenças Infecciosas e Parasitárias, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brazil, †Departamento de Biologia Celular e Molecular, Escola de Medicina de Ribeirão Preto da Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil, and ‡Laboratório de Micologia, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil

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 Cryptococcus 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].

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Correspondence: Mario León Silva-Vergara, Medicina Tropical, Caixa Postal 118, CEP: 38001-170, Uberaba, Minas Gerais, Brazil, Tel.: + 553433185286; Fax: + 553433185279; E-mail: marioleon.dip@mednet.com.br

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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 meningocencephalitis 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, MATα and MATα [17,18]. MATα 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 MATα 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)₄], 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 MATα, whereas the VGII MATα 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 thermostolerance 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 μL 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 μL 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 μL 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 μL TE (10 mM Tris–HCl, pH 7.5, 0.5 M EDTA, pH 8.0) containing 50 μg/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.

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Genotype determination by URA5-RFLP

Amplification reactions of the URA5 gene were performed in a final volume of 50 μL 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, 2 mM MgCl2, 1.5 U of Taq DNA polymerase and 50 ng of each primer URA5 (5′-ATGTCCTCCCAAGCCCTCGACTCG-3′) and SJ01 (5′-TTAAGACCTCTGAACCCAGTGACTACCTG-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 μL of the PCR amplicons was double digested with Sau96I (10 U/μL) (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 μL 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 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 μg/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 MATHaF (5′-CTTCACTGCCA-TCTTCACA-3′) and MATHaR (5′-GACCAAAGGTT-CATGCCA-3′). The a-mating type specific primers were MATaαF (5′-CGCCCTCTCAGCTACCTTCT-3′) and MATaαR (5′-AACGCAAGTAGAACTCGGGC-3′). Amplification reactions were performed in a final volume of 25 μL. 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 μL 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 (MATαα or MATαa, 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 μg/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 STE20αA gene sequence [37]. Amplification reactions were performed in a final volume of 50 μL, 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, 2 mM MgCl2, 1.5 U of Taq DNA polymerase and 50 ng of each primer JOHET264 (5′-AGCTGATGGCTGGATT-GAATAC-3′) and JOHET265 (5′-GTTCATATTCTCAGTACCTGTA-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 μg/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 MATα and ATCC 28958 MATα) 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, VNI/AFLP1A), WM 628 = LMM 796 (serotype AD, VNIII/AFLP2), WM 629 = LMM 797 (serotype D, VNI/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 diagnosed 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/μL) and HhaI (20 U/μL) 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).

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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

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>No. of strains</th>
<th>Canavamine-glycine-bromothymol blue (CGB)</th>
<th>Mating type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. neoformans</td>
<td>C. gattii</td>
<td>MATα</td>
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<tr>
<td>1999</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>5</td>
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<td>2000</td>
<td>9</td>
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<td>2006</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>5</td>
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<tr>
<td>2007</td>
<td>15</td>
<td>14</td>
<td>1</td>
<td>16</td>
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<tr>
<td>Total</td>
<td>81</td>
<td>76</td>
<td>5</td>
<td>81</td>
</tr>
</tbody>
</table>

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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, since 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.
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.

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