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Molecular typing of environmental *Cryptococcus neoformans*/*C. gattii* species complex isolates from Manaus, Amazonas, Brazil

Gleica Soyan Barbosa Alves,[1] Ana Karla Lima Freire,[2] Amaury dos Santos Bentes,[2] José Felipe de Souza Pinheiro,[3] João Vicente Braga de Souza,[4] Bodo Wanke,[5] Takeshi Matsuura[6] and Ani Beatriz Jackisch-Matsuura[7]

[1]*Programa de Pós-Graduação em Saúde Sociedade e Endemias na Amazônia – UFAM and CPqLMD/FIOCRUZ, Manaus, Amazonas, Brazil,* [2]*Programa de Pós-Graduação em Medicina Tropical, Universidade do Estado do Amazonas (UEA) and Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), Manaus, Amazonas, Brazil,* [3]*Centro de Controle de Zoonoses de Manaus, Manaus, Amazonas, Brazil,* [4]*Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil,* [5]*Laboratório de Micologia do Instituto Nacional de Infectologia Evandro Chagas (INI)/FIOCRUZ, Rio de Janeiro, Brazil,* [6]*Laboratório de Microbiologia, Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil and* [7]*Centro de Pesquisa Leônidas e Maria Deane (CPqLMD)/FIOCRUZ, Manaus, Amazonas, Brazil*

Summary

Cryptococcus neoformans and *Cryptococcus gattii* are the main causative agents of cryptococcosis, a systemic fungal disease that affects internal organs and skin, and which is acquired by inhalation of spores or encapsulated yeasts. It is currently known that the *C. neoformans*/*C. gattii* species complex has a worldwide distribution, however, some molecular types seem to prevail in certain regions. Few environmental studies of *Cryptococcus* have been conducted in the Brazilian Amazon. This is the first ecological study of the pathogenic fungi *C. neoformans*/*C. gattii* species complex in the urban area of Manaus, Amazonas, Brazil. A total of 506 samples from pigeon droppings ($n = 191$), captive bird droppings ($n = 60$) and tree hollows ($n = 255$) were collected from June 2012 to January 2014 at schools and public buildings, squares, pet shops, households, the zoo and the bus station. Samples were plated on niger seed agar (NSA) medium supplemented with chloramphenicol and incubated at 25°C for 5 days. Dark-brown colonies were isolated and tested for thermotolerance at 37°C, cycloheximide resistance and growth on canavanine-glycine-bromothymol blue agar. Molecular typing was done by PCR-RFLP. Susceptibility to the antifungal drugs amphotericin B, fluconazole, itraconazole and ketoconazole was tested using Etest® strips. In total, 13 positive samples were obtained: one tree hollow (*C. gattii* VGII), nine pigeon droppings (*C. neoformans* VNI) and three captive bird droppings (*C. neoformans* VNI). The environmental cryptococcal isolates found in this study were of the same molecular types as those responsible for infections in Manaus.

Key words: *Cryptococcus neoformans*, *Cryptococcus gattii*, environmental isolation, genotyping, Manaus, Amazonas, Brazil.

Introduction

Cryptococcosis is a major opportunistic fungal infection and is often fatal in immunocompromised individuals, especially those living with AIDS. Special attention only began to be paid to cryptococcosis in the 1980s, as the prevalence of the disease increased dramatically with the increasing incidence of AIDS.[1,2]

Correspondence: A. B. Jackisch Matsuura, Centro de Pesquisa Leônidas e Maria Deane (CPqLMD)/FIOCRUZ, Rua Teresina, 476, Adrianópolis, 69057-070 Manaus, Amazonas, Brazil.
Tel.: +55 92 3621-2333, +55 92 3621-2337. Fax: +55 92 3621-2377.
E-mail: ani@amazonia.fiocruz.br

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Members of the *Cryptococcus neoformans* and *Cryptococcus gattii* are the main causative agents of cryptococcosis. *C. neoformans* has worldwide distribution and can be isolated from various environmental sources, including soil, tree, birds dropping, especially pigeon droppings. *C. gattii* is traditionally cited as occurring in tropical and subtropical climates associated with tree species such as eucalyptus (*Eucalyptus camaldulensis*, *Eucalyptus tereticornis*), pottery tree (*Moquilea tomentosa*), pink shower (*Cassia grandis*) and fig (*Ficus microcarpa*).[3–7] Currently, there are several indications that this species can have a wider ecological range that previously recognised. However, the ecological niches of pathogenic species of *Cryptococcus* continue being investigated around the world.[8–19] The outbreak of *C. gattii* infection in 1999 in the temperate climate of Vancouver Island, British Columbia, Canada, led to a collaborative investigation to determine the extent of the outbreak and identify possible environmental reservoirs responsible for the outbreak.[20,21] In 2013, Hagen *et al.* [22] pointed to the Amazon rainforest as the geographical origin of *C. gattii* (VGII) outbreaks in Vancouver Island and the Pacific Northwest, USA.

In the last years, several molecular methods have been developed for genetic differentiation of *C. neoformans*/*C. gattii* species complex. Among them stand out fingerprinting PCR using specific primers for microsatellite (M13 or $(GACA)_4$), amplified fragment length polymorphism (AFLP) analysis, restriction fragment length polymorphism (RFLP) *URA5* analysis and multi-locus sequence typing (MLST). Regarding molecular types, *C. neoformans* has been grouped into AFLP1/VNI and AFLP1A/VNII (serotype A), AFLP3/VNIII (serotype AD) and AFLP2/VNIV (serotype D), whereas *C. gattii* has been grouped into types AFLP1/VGI, AFLP6/VGII, AFLP5/VGIII and AFLP7/AFLP10/VGIV (serotypes B and C).[23–29]

In 2015, a new taxonomy proposed the *C. neoformans* division into two species (*C. neoformans* and *Cryptococcus deneoformans*) and *C. gattii* into a total of five species (*C. gattii, Cryptococcus bacillisporus, Cryptococcus deuterogattii, Cryptococcus tetragattii* and *Cryptococcus decagattii*).[30] However, in this article, we are still distinguishing the two species as *C. neoformans* and *C. gattii*.

Cryptococcosis caused by *C. neoformans* (VNI) and *C. gattii* (VGII) has been reported in the state of Amazonas, northern Brazil, where infection with *C. neoformans* (VNII) has also been confirmed.[31] However, to date, no ecological study of *Cryptococcus* has been conducted.

This study aimed to determine the genetic diversity of *C. neoformans*/*C. gattii* species complex isolated from environmental samples collected in Manaus, Amazonas, Brazil. In addition, antifungal susceptibility testing was performed for at least one isolate from each administrative region in the city.

Materials and methods

Sample collection

A total of 506 samples were collected, of which 255 were from tree hollows, 191 from pigeon droppings and 60 from captive bird droppings. The samples were collected from June 2012 to January 2014 at schools and public buildings, hospitals, households, squares, parks, the bus station and the zoo. Approximately 30 g of each sample was placed in sterile a plastic bag, stored in a thermal box, and taken to the Laboratory of Biodiversity in Health, Instituto Leônidas e Maria Deane (Fundação Oswaldo Cruz), Manaus, Amazonas, Brazil, for processing.

Processing

Approximately 1 g of the sample was macerated in a mortar and pestle and transferred to a 125-ml Erlenmeyer flask containing 50 ml of 0.9% sterile saline. The mixture was agitated for 5 min and allowed to stand for 30 min. Next, 0.1 ml of the supernatant was plated onto Petri dishes containing Niger seed agar (NSA) supplemented with chloramphenicol (10 plates for each sample) and incubated at 25°C for 5 days. Plates were checked every 24 h. Smooth, moist, bright dark-brown colonies were subcultured on NSA plates for purification and subsequent identification. The estimated number of *Cryptococcus* propagules in each positive sample was expressed as colony-forming units per gramme of organic matter (CFU g^{-1}).

Identification of *Cryptococcus*

The isolated colonies were subcultured on Sabouraud's dextrose agar (SDA)for identification. Microscopy was performed using Indian ink (Nanquim Indian Ink) and lactophenol cotton blue staining. Thermotolerance at 37°C and cycloheximide resistance were also determined. Samples were identified by culturing the isolates on canavanine-glycine-bromothymol blue (CGB) agar.

DNA extraction

DNA extraction was done using silica-membrane-based purification with the QIAamp tissue kit and QIAamp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Molecular characterisation of fungal isolates

RFLP-URA5 and mating type

Genotyping of environmental isolates was performed using the RFLP-*URA5* method as described by Meyer *et al.* [26]. RFLP patterns were assigned visually by comparing them with the patterns obtained from standard strains. A set of eight laboratory standard strains representing each of the eight previously defined molecular types (WM148, molecular type VNI; WM626, molecular type VNII; WM628, molecular type VNIII; WM629, molecular type VNIV; WM179, molecular type VGI; WM178, molecular type VGII; WM161, molecular type VGIII; and WM779, molecular type VGIV) were used to assign molecular type and ensure reproducibility. In addition, the mating type of most isolates was also determined. Amplification of the gene for mating type was conducted in a final volume of 50 µl, and each reaction contained 1 × PCR buffer, 0.2 mmol l^{-1} dNTP, 50 ng of each primer [Mat-αF (5′-CTTCACTGCCATCTTCACCA-3′) and Mat-αR (5′-GACACAAAGGGTCATGCCA-3′), and Mat-aF (5′-CGCCTTCACTGCTACCTTCT-3′) and Mat-aR (5′-AACGCAAGAGTAAGTCGGGC-3′)], 3.0 mmol l^{-1} $MgCl_2$, and 2.5 U of Taq polymerase. PCR was performed as follows: initial denaturation at 94 C, 35 cycles of 30 s at 94 C, 30 s at 58C and 1 min at 72 C, followed by 7 min at 72 C and cooling down to room temperature.[32,33]

Antifungal susceptibility testing

Susceptibility to the antifungal drugs amphotericin B (AMB), fluconazole (FLZ), itraconazole (ITZ) and ketoconazole (KTZ) was determined using Etest® strips (bioMérieux, Marcy l'Étoile, France) and RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.5% (w/v) agar and 2% (w/v) glucose buffered to pH 7.0 ± 0.01 with 3-(*N*-morpholino) propanesulfonic acid (MOPS; Sigma-Aldrich). The inoculum was prepared from *Cryptococcus* colonies cultured in SDA after 72 h of growth and homogenised in 0.85% (w/v) NaCl to 1 McFarland standard. The plates were incubated at 35°C for 72 h. MIC (minimum inhibitory concentration) values were read from the test strips according to the manufacturer's instructions. *Candida parapsilosis* (ATCC 22019) was used as a quality control.

The obtained results were compared to those previously reported for other *C. neoformans*/*C. gattii* species complex isolates.[34–46]

Results

Of the 506 environmental samples collected from June 2012 to January 2014 across the six administrative regions in Manaus (Fig. 1), 13 *C. neoformans*/*C. gattii* species complex positive samples were obtained: nine pigeon droppings, three captive bird droppings ("Orange-fronted Yellow-finch" – *Sicalis columbiana*, "Chestnut-bellied Seedeater" – *Sporophila castaneiventris* and "Lined Seedeater" – *Sporophila lineola*) and one tree hollow (pottery tree – *Moquilea tomentosa*).

All isolates in the *C. neoformans*/*C. gattii* species complex were phenol oxidase-positive, able to grow at 37°C, and CGB-negative, except for the pottery tree isolate, which produced a blue colour on CGB agar. A total of 254 isolates were obtained, for which the estimated number of *Cryptococcus* propagules ranged from 52 to 7500 CFU g^{-1} of organic matter (Table 1).

The single isolate from *M. tomentosa* belonged to *C. gattii* (VGII), whereas all other isolates belonged to *C. neoformans* (VNI). In addition, all isolates were characterised being mating type α (Table 1).

Six *C. neoformans* isolates, one each from every administrative region in the city, and the single *C. gattii* isolate were selected for antifungal susceptibility testing. The susceptibility profiles of the selected cryptococcal isolates are shown in Table 2.

Discussion

Cryptococcus gattii (VGII) and *C. neoformans* (VNI) occupied different ecological niches, the former was isolated from a tree hollow while the latter was cultured from avian excreta.

C. gattii (VGII) was isolated from a tree hollow, whereas *C. neoformans* (VNI) was associated with avian excreta only. According to Nielsen *et al.*, [47] pigeon guano is not a suitable substrate for long-term survival of *C. gattii*, and the species is unable to sexually reproduce effectively on pigeon guano. Nevertheless, *C. gattii* has been isolated previously from excreta of several bird species, especially Psittaciformes.[48]

In Manaus, the occurrence of *C. gattii* was lower than that of *C. neoformans*. In this study, 255 samples from tree hollows and 251 samples from bird droppings (of pigeons and captive birds) were analysed,

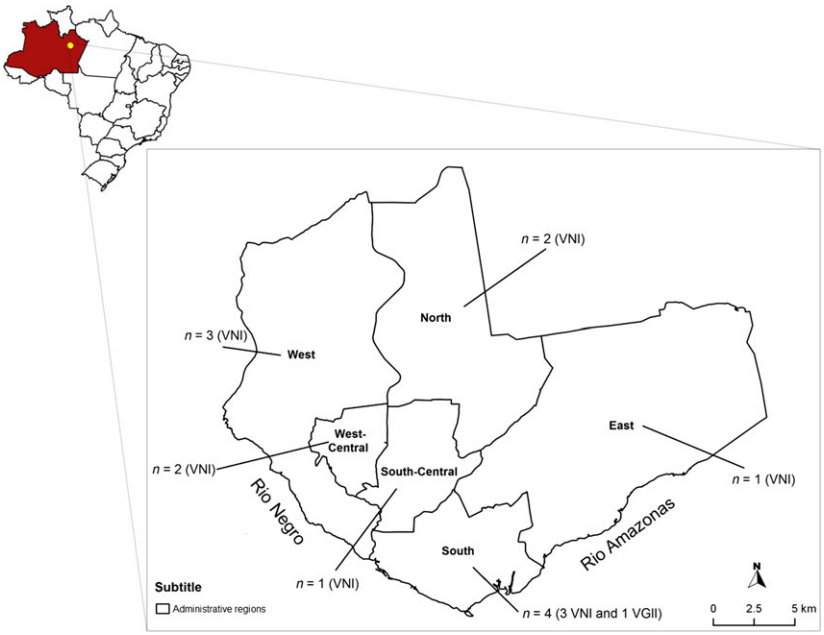

Figure 1 Map of Manaus, Amazonas, Brazil, showing the distribution of *Cryptococcus neoformans* (VNI) and *C. gattii* (VGII) among administrative regions of the city.

Table 1 Molecular type and CFU/g of environmental cryptococcal isolates from Manaus, Amazonas, Brazil.

Type of sample	Positive samples	Source	Species (molecular type)	CFU g^{-1}
Pigeon droppings (n = 191)	9	Food court	*C. neoformans* (VNI)	6500
		School 1	*C. neoformans* (VNI)	805
		School 2	*C. neoformans* (VNI)	57
		Square	*C. neoformans* (VNI)	4900
		Square	*C. neoformans* (VNI)	1500
		Square	*C. neoformans* (VNI)	1500
		Square	*C. neoformans* (VNI)	1500
		Public building	*C. neoformans* (VNI)	55
		Private company	*C. neoformans* (VNI)	52
Tree hollows (n = 255)	1	Pottery tree (*Moquilea tomentosa*)	*C. gattii* (VGII)	19
Captive bird droppings (n = 60)	3	*Sicalis columbiana*	*C. neoformans* (VNI)	950
		Sporophila castaneiventris	*C. neoformans* (VNI)	7500
		Sporophila lineola	*C. neoformans* (VNI)	4500

CFU, colony-forming unit.

resulting in positivity rates of 0.4% (1/255) and 4.8% (12/251) for *C. gattii* (VGII) and *C. neoformans* (VNI) respectively.

This is the first report of *C. gattii* isolated from *M. tomentosa* in northern Brazil after the report by Lazéra *et al.* [5] of *C. gattii* colonisation in a tree hollow of the same species at a public square in downtown Teresina, state of Piauí, northeastern Brazil. *M. tomentosa* is extensively used for afforestation of urban areas in large Brazilian cities. *C. gattii* (VGII) has also been isolated from *Guettarda acreana* in a forest reserve in the Amazon rainforest, northern Brazil and colonisation of other tree species such as *Senna siamea* and *Plathymenia reticulata* by *C. gattii* (VGII) in urban areas has also been reported.[16,49,50] As in the reports by Anzai *et al.* [16] and Costa *et al.*, [50] the *C. gattii* isolate in our study was obtained from a tree hollow located in an urban area.

Cryptococcus neoformans (VNI) was isolated in 4.8% of bird dropping samples analysed. A similar positivity rate (6.6%, 8/122) was reported by Takahara *et al.* [51] in environmental samples from Cuiabá, state of Mato Grosso, midwestern Brazil, in the southern part of the Brazilian Amazon. The authors suggested that the high temperatures in the study area inhibit the growth of *C. neoformans*, which may explain the low

Table 2 Antifungal susceptibility of environmental cryptococcal isolates from Manaus, Amazonas, Brazil.

Antifungal		A	B	C	D	E	[1]F	G
Amphotericin B 0.002 32 μg $(ml)^{-1}$	AMB	0.19	0.25	0.125	0.19	0.19	0.125	0.19
Fluconazole 0.016 256 μg $(ml)^{-1}$	FLZ	2	6	4	4	4	48	24
Itraconazole 0.002 32 μg $(ml)^{-1}$	ITZ	0.125	0.5	0.19	0.25	0.38	2	0.5
Ketoconazole 0.002 32 μg $(ml)^{-1}$	KTZ	0.047	0.094	0.047	0.125	0.064	0.75	0.094

One of the *C. neoformans* (VNI) isolates was not able to grow at 35°C in RPMI 1640 + MOPS medium and had its susceptibility profile determined at 28°C. AMB, amphotericin B; FLZ, fluconazole; ITZ, itraconazole; KTZ, ketoconazole; A-F, *C. neoformans* (VNI) isolates; G, *Cryptococcus gattii* (VGII) isolate.

[1]MIC values determined at 28°C.

detection rates observed in that study. In this study, *C. neoformans* was isolated mainly from locations such as squares and schools, where a large number of people, especially children, are constantly exposed to propagules. In fact, schools provide abundant resources for pigeons due to their architectural design and the fact that their air-conditioning systems are poorly protected from birds.

In Brazil, all genotypes of the *C. neoformans*/*C. gattii* species complex have been isolated from environment, except the genotype VGIV. The most frequently reported types are VNI and VGII,[52,53] which are also the main molecular types responsible for infections in the state of Amazonas. In a study conducted from 2006 to 2008 that analysed 40 clinical isolates, 31 (75.5%) belonged to *C. neoformans* (VNI) and nine (22.5%) to *C. gattii* (VGII).[54] A similar study that analysed 57 clinical isolates from 2006 to 2010 reported that 68.42% (39/57) belonged to *C. neoformans* (VNI), 29.83% (17/57) to *C. gattii* (VGII) and 1.75% (1/57) to *C. neoformans* (VNII).[17] *C. gattii* (VGII) has been responsible for severe cases of meningitis in HIV-negative individuals in the state of Pará, including a large number of children, and thus deserves special attention in northern Brazil.[33,55]

Antifungal susceptibility testing for AMB, FLZ, ITZ and KTZ revealed differential results susceptibility of *C. neoformans* isolates to the same antifungal agents (Table 2), demonstrating the inherent variability in isolates across sites. The *C. gattii* (VGII) isolate exhibited higher susceptibility to AMB and to KTZ than have other clinical and environmental *C. gattii* isolates from Brazil,[35–37] but its susceptibility to those drugs was similar to that of isolates from other countries.[38–40] Resistance to FLZ has previously been reported for *C. gattii*, and was expected to be observed in this study.[37,41] Susceptibility of *C. neoformans* isolates was similar to that of other environmental isolates from Brazil,[42,43] but susceptibility values for FLZ, ITZ and KTZ were higher than those of environmental isolates from other countries.[38,39,44]

Currently, CLSI breakpoints for *C. neoformans*/*C. gattii* species complex are not well established to determine if isolates are sensitive and/or resistant. Espinel-Ingroff *et al.* [45,46] proposes the adoption of species-specific values and the genotype of the investigated isolates. In light of this proposal, all our isolates are sensitive to the tested antifungals.

It must be noted, however, that despite the importance of environmental studies for determining the circulating genotypes in the country, the small number of environmental isolates from Brazil that have been reported hinders valid and comprehensive comparisons of antifungal susceptibility across regions and between countries.

In conclusion, *Cryptococcus neoformans* (VNI) was widely distributed across Manaus, and the environmental cryptococcal isolates identified in this study were of the same molecular types as those responsible for infections in that city. This study reveals possible environmental reservoirs from which the human population of Manaus may be exposed to *C. neoformans* (VNI) and *C. gattii* (VGII), and may help public health authorities design strategies to educate the population to prevent cryptococcal infection.

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Conflict of interest

None.

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