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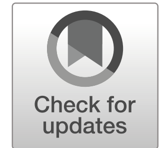

ENVIRONMETAL MICROBIOLOGY - RESEARCH PAPER

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Cryptococcus gattii VGII isolated from native forest and river in Northern Brazil

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Abstract

Background Cryptococcosis is a global invasive mycosis associated with significant morbidity and mortality. In the northern region of Brazil, this disease is caused by *Cryptococcus neoformans* genotype VNI and *Cryptococcus gattii* genotype VGII. However, few environmental studies have been conducted in this large tropical area.

Aims This study was performed to isolate, genotype, and determine the frequency of cryptococcal agents in environmental samples near Manaus, Amazonas, Brazil.

Methods A total of 970 environmental samples (290 from soil, 290 from decaying plants, 5 from insects, 280 from the Negro river, and 105 from small streams within the city of Manaus) were collected and plated on Niger seed agar. In addition, 20 subcultures obtained from each positive sample were analyzed by PCR-RFLP (URA5) and PCR for genotyping and determination of mating type.

Results Six samples were positive for isolates from the *C. gattii* species complex. Of those, three samples were from Adolpho Ducke Forest Reserve and three were from the Negro river. All isolates were *C. gattii* genotype VGII (mating type *MAT*α).

Conclusion Genotype VGII proved to be the most important genotype found in the environmental samples. The genotype VGII has been described as one of the most virulent and less susceptible to antifungals and responsible for important outbreaks. This is the first study to demonstrate isolation of *C. gattii* (VGII) from the Negro river.

Keywords Amazon region · *Cryptococcus* · Genotyping · Isolation · M13 PCR fingerprinting · PCR-RFLP of *URA5*

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Introduction

Cryptococcosis is an opportunistic invasive mycosis that occurs globally with 223,100 cases estimated to occur yearly and a mortality rate of about 81% [1, 2]. It is caused by the *Cryptococcus neoformans* species complex and the *Cryptococcus gattii* species complex with the following genotypes: VNI/AFLP1, VNII/AFLP1A, VNIII/AFLP3, VNIV/AFLP2, VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, and VGIV/AFLP7 [3–6]. Understanding the distribution of these agents in the environment is important for prevention of outbreaks since the infection is acquired through inhalation of infectious propagules from the environment [7]. Previous works have investigated the presence of these microorganisms in samples such as soil, air, water, animals, and insects [8, 9].

In the state of Amazonas (Northern Brazil), cryptococcosis is caused by *C. neoformans* VNI and *C. gattii* VGII affecting approximately 30–40 patients per year. The majority of the

patients are male (73%), the mean age is 39.8 (range of 19–68 years), and most cases are from Manaus (83%). HIV infection is reported for a majority of the patients (87%), neurocryptococcosis is the most frequent clinical presentation (97%), and mortality is around 50%. In addition, at least three children are affected every year [10–12].

In a recent report, isolates from molecular types VNI/VGII were obtained from pigeon droppings and captive bird droppings and from tree hollow environmental samples from urban areas of the city of Manaus [9]. Manaus is surrounded by both native forests and numerous rivers and their tributaries. Studies on the distribution of these agents in native forests (soil samples, rotting plant material, insects) and in water (anthropogenically impacted or not) near the urban areas of Manaus are necessary. Thus, this study aimed to isolate and genotype *C. neoformans* and *C. gattii* isolates from environmental samples.

Materials and methods

Microorganisms

Cryptococcus 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 as references in the genotyping assays. These strains were recommended by Meyer et al. [4] as references for *Cryptococcus* fingerprint. These strains were obtained from the mycological collection of FIOCRUZ, Rio de Janeiro, Brazil.

Sample collection

Samples (soil, decaying wood, insects, and water) were collected from March to December 2013 at three sites: (a) Adolpho Ducke Forest Reserve, (b) Negro river, and (c) small streams.

Specific collection sites

(a) Adolpho Ducke Forest Reserve This forest reserve belongs to the Instituto Nacional de Pesquisas da Amazônia (INPA). An area of 100 km^2 which surrounds the city of Manaus is still largely untouched and was surveyed. In this area (1000 m^2, central point south latitude 2° 91′ 97″ and east longitude 59° 97′ 98″), 585 environmental samples were collected, including 290 soil samples (1 g each, 2 cm deep); 290 samples (1 g each) of plant material on decaying wood (samples from the ground, in plant folds, and in tree hollows); and 5 samples of ants (*Atta sexdens*). All samples were placed in sterile plastic bags. These samples were collected in May 2013.

(b) Negro river and Tarumã River Two hundred eighty water samples were collected (30 cm deep), between 5 to 50 m from the riverbank. The first sample was collected at south latitude 3° 00′ 57″ and east longitude 60° 09′ 98″, and the last sample was collected at south latitude 3° 12′ 96″ and east longitude − 59° 93′ 70″ along a 29 km stretch. The volume of each sample was 15 mL, and the length of each collection site was 100 m. These samples were collected (three times) from March to July 2013.

(c) Seven igarapés (streams) Fifteen samples were collected from each of the following sites: Petrópolis (− 3° 12′ 23″; 59° 99′ 18″), Quarenta (− 3° 13′ 30″; 60° 00′ 11″), Cachoerinha (− 3° 12′ 01″; 60° 00′ 04″), Mindú (− 3° 09′ 97″; 60° 02′ 26″), Sambódromo (− 3° 09′ 02″; 60° 02′ 95″), Tarumã (− 3° 00′ 90″; 60° 05′ 52″), and Tancredo Neves (− 3° 04′ 77″; 59° 94′ 55″), totaling 105 water samples. These samples were collected (three times) from August to November 2013.

Isolation of Cryptococcus spp.

Decaying wood and soil samples Samples were obtained from the ground, in plant folds, and in tree hollows. Sample processing was performed according to the protocols of Lazéra et al.[13] and Passoni et al.[14] with modifications. Samples (1 g of material) were macerated in a sterilized mortar and pestle and suspended in 49 mL of solution (9% saline, 80% Tween, 0.2 g chloramphenicol, and 0.2 g amikacin). This was followed by shaking for 5 min; after 30 min decantation, a 100-μL sample was transferred to a petri dish containing Niger seed agar (NSA).

Water samples Samples were centrifuged at 6000*g* for 10 min. The supernatant was discarded, and 0.1 mL of the liquid/slurry was transferred to a petri dish.

The samples were plated individually on petri dishes (one petri dish per sample) containing Niger Seed Agar (NSA) (*Guizotia abyssinica* 50 g/L, glucose 1 g/L, KH_2PO_4 1 g/L, creatinine 1 g/L, agar 15 g/L, penicillin G 40 units/L, and gentamicin 80 mg/L). All culture media were placed at room temperature (25 °C). Colonies that showed a dark brown color on NSA were analyzed and subsequently subjected to purification and phenotypic identification via melanin production and urease production and canavanine glycine bromothymol blue (CGB) [15–17]. Isolates were cryogenically preserved in mineral oil at − 70 °C and then deposited in the “microorganisms of medical interest” collection of the INPA Mycology Laboratory.

Genotyping

PCR fingerprinting

This protocol employed a sequence specific for the M13 minisatellite (5′-GAGGGTGGCGGTTCT-3′) [4]. The

amplification reaction was performed in a final volume of 25 µL. Each reaction contained 10 ng of DNA template, buffer solution (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM 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, 2 min extension at 72 °C, and a final extension of 6 min at 72 °C. 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 [4]. Each reaction contained 10 ng of template DNA, buffer (10 mM^{-1} Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 50 ng of URA5 primer (5′-ATGTCCTCCCAAGCCCTCGACTCCG-3′), 50 ng of SJ01 primer (5′-TTAAGACCTCTGAACACCGTACTC-3′), and 1.5 U of recombinant Taq DNA polymerase (Invitrogen). PCR was performed in a Verite 96 thermocycler (Applied Biosystems). Reactions 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. Size and purity of PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with Sybr Green and illuminated with ultraviolet light. Next, 8 µL of each PCR product was mixed with 1-mL buffer, then was digested with restriction endonucleases *Sau96I* (10 U) and *HhaI* (20 U) for 3 h or overnight at 37 °C. PCR products were visualized by electrophoresis on a 3% agarose gel and stained with SYBR® Green (SYBR Safe DNA Gel Stain, Invitrogen, Carlsbad, USA).

Mating types

Mating types were determined by PCR [4], final volume of 25 µl. The α-type primers were Mat- α F(5′-CTTCACTGCCATCTTCACCA-3′) and Mat- α R(5′-GAC-ACAAAGGGTCATGCCA-3′), and the a-type primers were Mat-aF (5′-CGCCTTCACTGCTACCTTCT-3′) and Mat-aR (5AACGCAAGAGTAAGTCGGGC-3′). Each PCR reaction contained 20 ng of template DNA, buffer solution (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 20 ng of each primer, and 1.5 U recombinant Taq DNA polymerase (Invitrogen). PCR products were visualized by electrophoresis on a 2% agarose gel and stained with SYBR® Green (SYBR Safe DNA Gel Stain, Invitrogen, Carlsbad, USA).

Results

Six samples were positive for the presence of the *C. gattii* species complex (Table 1): two (2/290) from decaying wood samples, one (1/5) from insects (*Atta sexdens*), and three (3/280) from the Negro and Tarumã Rivers. Soil (290) and stream polluted water (105) samples were negative for the presence of *Cryptococcus* spp.

Molecular assays were performed to determine the genotypes of the isolates and to investigate the presence of more than one genotype per sample investigated. For each positive sample ($n = 6$), 20 colony-forming units (CFUs) were isolated, purified, and assayed for genotype. All 120 CFUs isolated belonged to the *C. gattii* species complex from genotype VGII and mating type *MAT*α (Table 1).

Discussion

Causative agents of cryptococcosis have been isolated from different environmental sources and represent reservoirs for infection of humans and animals [8, 18, 19]. In Northern Brazil, cryptococcosis is found to be endemic and is associated with *C. neoformans* (genotype VNI) and *C. gattii* (genotype VGII) [10, 20, 21]. The present work detected *C. gattii* (VGII) in Amazonian environmental samples and is the first study to demonstrate the isolation of this species from water samples from the Negro river.

Studies carried out worldwide have shown that the same cryptococcal genotypes are found in both patients and environmental sources from the same location [7, 22, 23]. When Ferreira et al. [24] evaluated the genotypic diversity of environmental isolates occurring in Northern Portugal, they found that *C. neoformans* of the VNI genotype was common among environmental and clinical samples. The results obtained by Frases et al. [25] in Spain showed the prevalence of the *C. neoformans* VNIII genotype among isolates from pigeon feces and a large presence of this genotype among clinical isolates. In Australia and at Vancouver Island, where the prevalence of cryptococcosis is high, the isolation of *Cryptococcus* species from environmental sources is frequent, and the genotypes were found to be identical to those of clinical isolates [21, 26]. Previous studies showed that the most important clinical genotypes in Amazonas are the VNI and VGII strains [10, 20]. Our research demonstrates that genotype VGII can be isolated from environmental samples. However, no genotype VNI was isolated in the present work. In fact, the literature shows that *C. gattii* is more closely associated with samples of decaying vegetation and *C. neoformans* is associated with birds excreta [10, 20, 21].

In the present work, *C. gattii* genotype VGII was isolated from decaying wood. The quantities of isolates obtained from each of our samples were similar to those observed

Table 1 Isolation of microorganisms from the *Cryptococcus gattii* species complex from samples of native forest, the Negro river, and small streams crossing and surrounding the city of Manaus, Amazonas, Brazil

Collection sites	Type of samples	Number of samples	Positive samples	Global Positioning System of the positive samples	Concentration in CFU/g (soil) or CFU/mL × 10^3 (water)	Fingerprinting PCR-RFLP URA5	Mating type
Ducke Reserve	Decaying wood	290	2	2° 91′ 97″ S, 59° 97′ 96.0″ W	750	VGII	*MATα*
				2° 91′ 99″ S, 59° 97′ 99.7″ W	123	VGII	*MATα*
	Soil	290	0		–	–	–
	Insects	5	1	2° 91′ 80″ S 59° 98′ 00.0″ W	65	VGII	*MATα*
Rio Negro/Tarumã River	Water	280	3	3° 07′ 45.9″ S 60° 02′ 22.5″ W	440	VGII	*MATα*
				3° 08′ 28.3″ S 60° 01′ 24.2″ W	44	VGII	*MATα*
				3°08′ 28.7″ S 60° 01′ 24.0″ W	65	VGII	*MATα*
Polluted streams crossing Manaus	Water	105	0	–	–	–	–

in other studies of tropical forests [27–29]. *C. gattii* genotype VGII seems to be adapted to this kind of substrate. The only other study in a wild area of an Amazon rainforest was performed in the Maracá Island, where only one of the 260 samples collected from 148 hollow trees was positive for *C. gattii* [30]. A focal distribution of primary habitats associated with the low relative abundance in this wild environment may have reduced the chance of *Cryptococcus* detection. The overgrowth of fast-growing filamentous fungi observed in the seeded samples did not favor visual screening of *C. gattii* colonies, mainly when a low number of colonies of *Cryptococcus* are present in the collected sample material. These factors possibly lead to false negative results when using the NSA plating method. Moreover, the low positivity observed does not exclude the possible role of *C. gattii* in wild environments. Climatic changes, human-induced land use, and trade of native wood from the Amazon rainforest are possible drivers of geographical dispersal of propagules, and consequently, disease emergence events from original habitats in wild forests.

One isolate was isolated from an ant of the species *Atta sexdens*. The insect was collected alive carrying propagules of *C. gattii* genotype VGII suggesting it could be introduced to new locations through passive transport. The literature is poor about the isolation of *Cryptococcus* from insects, however, these microorganisms were isolated from insect frass [31], ants [32], and bees 33. More studies should be done to study the importance of the insects in the dissemination of isolates from the *C. neoformans/C. gattii* species complex in the environment.

In the present work, we did not find *C. neoformans* and *C. gattii* from soil samples. Kidd et al. [21] analyzed 77 samples of soil from British Columbia, Canada, and found only two samples positive for the *C. gattii* species complex. Low rates of microbial isolation were also experienced by Yamamura et al. [34] They analyzed 120 soil samples and plant material in the city of Londrina (Paraná-Brazil) and only one sample was positive for *C. gatiii*.

This study is the first to isolate *C. gattii* (VGII genotype) from water samples taken from the Negro river. Specifically, in the waters of the Negro river, this microorganism finds an adequate environment (temperature 25 °C and pH 5.0) and phenolic compounds derived from decaying wood in the river as metabolic substrates [35]. The Negro river may be a source/distributor of propagules where they can survive for as much as a year [21]. Just one previous study had isolated microorganisms of this species complex from water samples with frequencies as high as 27 positives per 132 samples, as reported by Kidd et al. [21]. These results suggest that more studies that include water samples and evaluate the importance of water as a dispersal agent are necessary. The absence of isolates from the waters from igarapés (small streams) that cross Manaus may be due to a high level of organic matter, high pH (8,0), and low oxygen content [35, 36].

Regarding sexual or mating type, all 120 of the isolates analyzed were of sexual type *MATα*. The organisms of this sexual type are prevalent in clinical and environmental samples. This higher prevalence of the α mating type may be due to the selective advantages of longer survival in the environment and greater virulence [34, 37, 38]. Specifically in our region, these results agree with the clinical findings of Freire et al. [12], who investigated the mating types of the yeasts that cause cryptococcosis.

The low positivity observed in environmental samples is similar to that found in other works that investigated cryptococcosis agents in forest with low anthropogenic impact [21, 30]. This result can be related to the preservation of the native biodiversity and/or microbial competition, however, more studies are necessary to understand the microbial dynamics in these environments.

C. gattii VGII is considered a primary pathogen less susceptible (conventional antifungals) and virulent according to some studies [39]. This genotype (VGII) caused the Vancouver outbreak and recent phylogenetic studies suggest that South America may be the source of this microorganism [8, 40]. Even at a low density, the occurrence of *C. gattii*

species complex VGII in the Forest Reserve (Adolpho Ducke) may represent a source of the organism's propagules from native forest to the city. This is reinforced by studies that isolated *C. gattii* species complex VGII from household dust in the nearby cities of Santa Isabel and Iranduba in Amazonas [41] suggesting that *C. gattii* species complex is well dispersed in the Amazon as a potential source of this pathogen for urban environments [21]. In experimental conditions, no isolates from *C. neoformans* species complex were obtained. This result was expected since previous works have demonstrated that *C. neoformans* is mainly isolated from birds excreta, while *C. gattii* species complex is mainly isolated from samples related to plant debris [9, 21] .

This is the first study to demonstrate that the causative microbial genotype (VGII) of cryptococcosis occurring near Manaus can be isolated from environmental samples including waters of the Negro river. Further research of the etiologic agents of cryptococcosis from environmental samples can inform us more about the threat they pose to human health.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

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