



***Cryptococcus gattii* VGI and *Cryptococcus neoformans* VNI Associated with Wood Decay in Ficus Hollow Trees in Rio de Janeiro, Brazil**

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Authors' contributions

This work was carried out in collaboration between all authors. Study design was performed by authors MSL, GGB and LT; the environmental samples were collected and analyzed by authors GGB and LT; results analysis were performed by authors MSL, GGB, LT and BW; the first draft of the manuscript was written by authors GGB and MSL. All authors read and approved the final manuscript.

Research Article

Received 30th November 2012
Accepted 1st February 2013
Published 23rd February 2013

ABSTRACT

Aim: Considering the geographic expansion of *Cryptococcus gattii*, the aim of this study was to investigate hollows of living trees as a reservoir of *C. gattii* in Rio de Janeiro, Brazil.
Place and Duration of the Study: In an urban quarter of Rio de Janeiro city, 80 samples of decaying wood were collected. In addition, 85 decaying wood samples were collected in the wild rainforest. The samples were analyzed at the Mycology Laboratory, Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation, from 2008-2010.
Methodology: Samples were collected by scraping the inner decaying wood of the hollows of the trunks of each tree. Pathogenic *Cryptococcus* species were identified by: brown colonies on niger seed agar (NSA) medium, thermotolerance at 35°C, cycloheximide sensitivity, carbon and nitrogen assimilation tests performed by 32-Vitek System (Vitek ICB, bioMeriux, Durham, EUA). Canavanine-glycine-bromothymol blue medium (CGB) was used to determine the species of the isolates and the genotypes were determined by restriction fragment length polymorphism of *URA5* gene.
Results: After plating the samples on NSA, 584 colonies were obtained from the urban quarter. *C. gattii* VGI was identified in 98% of colonies, followed by *C. neoformans* VNI 2%.

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The positivity of the urban area was 7.8%. The concentrations of the fungi in hollows of ficus trees ranged from 50 to 56,250 colony-forming units per gram of sample (CFU/g).

Conclusions: For the first time in Rio de Janeiro *C. gattii* VGI was isolated in a hollow of living tree.

Keywords: Cryptococcosis; *Cryptococcus gattii*; molecular type VGI; urban area.

1. INTRODUCTION

Cryptococcosis is a world-wide emerging systemic mycosis which remains potentially fatal unless diagnosed early. The agents *Cryptococcus neoformans* (genotypes VNI-VNIV) and *Cryptococcus gattii* (genotypes VGI-VGIV) are the main agents of severe meningoencephalitis in, respectively, immunocompromised and immunocompetent hosts. The infection is acquired by inhalation of aerial propagules that cause a primary lung infection, which usually has a spontaneous regressive course. However, few cases may disseminate progressively to the central nervous system [1].

Cryptococcus neoformans occurs globally, whereas *C. gattii* prevails in tropical and subtropical areas. Initially, pigeon habitats were investigated to detect environmental sources of *C. neoformans*, the most frequent agent of cryptococcosis. The intensive and exclusive investigation of pigeon habitats on continents lead to the ignorance of the primary natural habitat of the agents of cryptococcosis for a long time. *C. gattii* has been repeatedly isolated from decaying wood substrates in trees, sometimes along with *C. neoformans*, recognizing a new natural habitat for the agents of cryptococcosis [2,3]. At first, *Eucalyptus* trees had been the main target for the environmental studies of *C. gattii* and the association of the fungi and *Eucalyptus* trees seemed to be specific. But, on the contrary, now it is clear that *C. gattii* and *C. neoformans* are associated with native and introduced trees wherever these agents became established, allowing a more comprehensive view on their ecology. The roster of tree species from which *C. gattii* and *C. neoformans* have been isolated is still increasing, reflecting geographic patterns and possible changes in their regional distribution [4]. Many different genera of trees, natives or adapted, were reported to be found associated with *C. gattii* in Argentina and Colombia (15 genera of trees); Canada, Mexico and United States (9 genera of trees); India (11 genera of trees); Australia (2 genera of trees); and Spain and Netherlands (4 genera of trees) [5,6,7]. In Brazil, the following trees were reported to be associated with each agent:

1. ***C. neoformans*:** *Anadenanthera peregrine*, *Caesalpinia peltophoroides*, *Cassia grandis*, *Eucalyptus camaldulensis*, *Eucalyptus tereticornis*, *Eucalyptus* spp., *Ficus microcarpa*, *Guettarda acreana*, *Licania (Moquilea) tomentosa*, *Myroxylon peruiferum*, *Senna multijuga*, *Senna siamea*, *Syzygium cumini (S. jambolanum)* and *Theobroma cacao*;
2. ***C. gattii*:** *Cassia grandis*, *Eucalyptus camaldulensis*, *Eucalyptus* spp., *Ficus microcarpa*, *Guettarda acreana*, *Licania (Moquilea) tomentosa* and *Senna siamea* [4].

While *C. neoformans* genotype VNI is the predominant agent of cryptococcosis worldwide, *C. gattii* genotypes VGI and VGII prevail as agent of cryptococcosis in immunocompetent hosts. *C. gattii* genotype VGI have been isolated from the environment in Argentina, Brazil, Colombia, United States, Portugal, India and Australia [9,10,11,12,15]. However, since 1999,

C. gattii genotype VGII is the main agent of the cryptococcosis outbreak in Vancouver, British Columbia, a temperate area, expanding to the Pacific Northwest of the United States and has been isolated from different species of native trees [13].

Two major epidemiological trends of the mycosis are observed in Brazil: *C. neoformans* VNI (serotype A) infection occurs predominantly in southern regions (S and SE regions); *C. gattii* VGII (serotype B) infection occurs predominantly in northern regions (N and NE regions), endemic in the Amazon and semi-arid NE regions [14,15,16]. This pattern of distribution is corroborated by the isolation of these agents from several environmental sources, including host trees [15]. Previous environmental studies in the city of Rio de Janeiro, tropical semi-humid region in SE, demonstrated the predominance of *C. neoformans* VNI in bird habitats, domestic dust, as well as in hollow trees of *Cassia grandis*, *Senna multijuga* and *Ficus microcarpa* [8,2,17,18,15].

The occurrence of autochthonous cases of meningoencephalitis in children caused by *C. gattii* VGII [19] in Rio de Janeiro suggests a geographic expansion of this agent in the northern-southern direction of Brazil. Therefore, we investigated hollows of living trees as a reservoir of *C. gattii* in Rio de Janeiro, Brazil.

2. MATERIALS AND METHODS

2.1 Study Areas and Trees

From 2008 to 2010, a total of 165 living trees with decomposing wood in their hollows were selected for investigation in two distinct areas of Rio de Janeiro: 1) Eighty trees located in the district of Botafogo, in front of the Botafogo Bay, 78 of them were *Ficus microcarpa* and, two *Licania tomentosa*, distributed as follows: Canoinhas square (5), Nicaragua square (15), Morro da Viuva block (35) and Marinha do Brasil square (25); 2) 85 trees located in the Atlantic Forest Park (CFMA) of the Campus of FIOCRUZ at the district of Jacarepagua, an environmental reserve of Atlantic Forest in Rio de Janeiro (Fig. 1).

2.2 Sampling and Processing

Samples were collected by scraping the inner decaying wood of the hollows of the trunks of each tree. The samples were processed according to [2]: after homogenization, 1g of each sample was suspended in 50 ml sterile physiological saline with chloramphenicol, vigorously shaken, and allowed to settle for 30 min. The supernatant (0,1 mL) was inoculated onto 10 plates with niger seed agar (NSA) medium with chloramphenicol and amikacin, incubated at 25°C and observed regularly up to 5 days. Colony-forming units per gram of sample (CFU/g) were assessed by counting the brown colonies grown onto NSA medium.

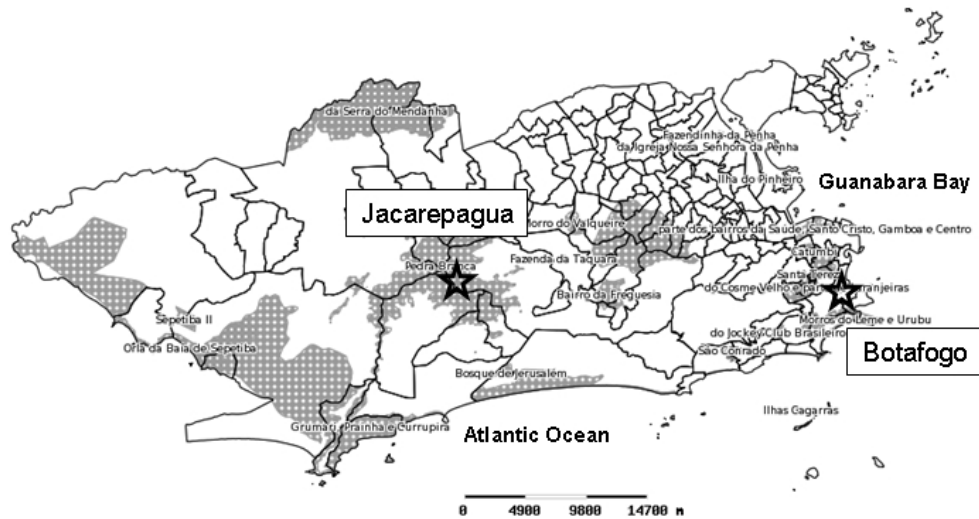


Fig. 1. Map of Rio de Janeiro city. The gray areas represent the environmental reserves; the stars show the sampling locations which are approximately 30 Km distance from each other

2.3 Identification of Isolates

Pathogenic *Cryptococcus* species were identified by: brown colonies on NSA medium, thermotolerance at 35°C, cycloheximide sensitivity, carbon and nitrogen assimilation tests performed by 32-Vitek System (Vitek ICB, bioMeriux, Durham, EUA). Canavanine-glycine-bromothymol blue medium (CGB) was used to determine the species of the isolates.

2.4 Molecular Identification

The genotypes VNI and VGI were identified by *URA5*-RFLP according [20]. The PCR was performed directly from the colony according to a protocol described by [21] with some modifications: small amount of cells from a single colony grown on Sabouraud-dextrose medium for 2 days at 25°C was transferred into 25 µL of master mix with the aid of a tip. The master mix contained 1X PCR buffer [2 mM Tris-HCl (pH 8.4), 5 mM KCl - Invitrogen], 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH), 3 mM magnesium chloride, 1.5 U Platinum[®] *Taq* DNA polymerase (Invitrogen), and 50 ng of each primer *URA5* (5' ATGTCCTCCCAAGCCCTCGACTCCG 3') and *SJ01* (5' TTAAGACCTCTGAACACCGTACTC 3'). PCR was performed for 35 cycles as follows, at 94°C with a 2 min initial denaturation, 45 s denaturation at 94°C, 1 min annealing at 61°C, 2 min extension at 72°C, and final extension cycle for 10 min at 72°C. A total of 22 µl of PCR products were double digested with *Sau96I* (5U/µl) and *HhaI* (20U/µl) for 3 h, and the fragments were separated by 3% agarose gel electrophoresis at 100 V. RFLP patterns were assigned visually by comparison with patterns obtained from standard strains VNI (CFP 55/WM 148), VNII (CFP 56/WM 626), VNIII (CFP 57/WM 628), VNIV (CFP 58/WM 629), VGI (CFP 59/WM 179), VGII (CFP 60/WM 178), VGIII (CFP 61/WM 161) e VGIV (CFP 62/WM 779) [20] stored in the Culture Collection of Pathogenic Fungi (CFP), FIOCRUZ, Brazil.

2.5 Statistical Analysis

Statistical analysis of 584 *C. gattii* and *C. neoformans* isolates was performed using a *P*-value = .05 to define significance. Univariate analysis was performed using chi-square test.

3. RESULTS AND DISCUSSION

In the Atlantic Forest Park (CFMA) all the trees studied were negative for pathogenic *Cryptococcus* species. In the urban area, seven *Ficus microcarpa* trees were positive (7/80; 8.75%). A total of 584 phenoloxidase positive colonies were identified: 572 *C. gattii* VGI and 12 *C. neoformans* VNI. *C. gattii* VGI was identified alone in six trees, and in one of these trees this genotype was co-isolated with *C. neoformans* VNI (Table 1, Fig. 2).

Table 1. *Cryptococcus gattii* and *C. neoformans* in decaying wood in different trees in the city of Rio de Janeiro, SE Brazil

Place	Positive/ examined trees	CFU/g	Nº of colonies identified	<i>C. gattii</i> VGI	<i>C. neoformans</i> VNI
Canoinhas square	1/05	56.250	300	288	12
Nicarágua square	1/15	100	2	2	0
Morro da viúva block	1/35	15.700	200	200	0
Marinha do Brasil square	4/25	650	13	13	0
		950	19	19	0
		1.400	28	28	0
		1.600	32	32	0
Total	7/80	-	584	572	12

*Number of phenol-oxidase positive colonies per gram of sample in each positive tree.

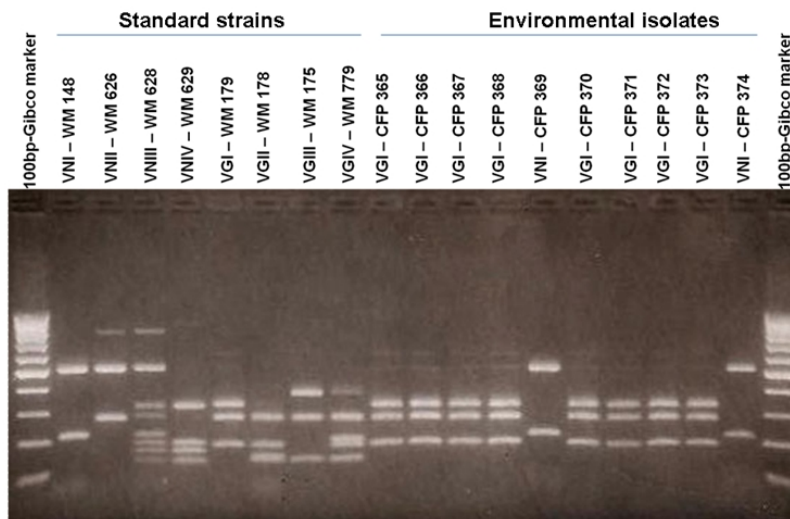


Fig. 2. Molecular typing profiles generated by restriction fragment length polymorphism (RFLP) analysis of *URA5* gene from standard strains and from representative environmental samples

The possibility of the occurrence of the agents of cryptococcosis was 5.8 fold higher in urban than in wild environment (chi-square test; P -value = .008). The genotype *C. gattii* VGI prevailed in all trees, including the one in which the genotype VNI was co-isolated.

From the environmental and ecological standpoint we studied two distinct areas in Rio: 1) Botafogo, a densely populated urban district with a heavy anthropogenic influence, marked by big buildings concentrated in landfill areas previously bathed by the sea and subsequently planted with trees for landscaping. 2) CFMA, a wild preserved area, with minimal anthropogenic influence. Despite the 85 hollows analyzed all of them were negative to *C. neoformans* and *C. gattii*. In contrast, the urban area showed positivity of 8.75% (7/80).

The tolerance to high temperature and adaptation to rapid environmental changes seems to be an important behavior of microorganisms to adapt to an urban environment. Big cities are like heat islands, registering average temperatures up to 12°C higher than outside the urban perimeter [22]. The agents of cryptococcosis are thermotolerant in the range of 35 to 37°C. Other microorganisms less adapted to environmental changes, e.g. the rising of temperatures, are usually less pathogenic to mammalian hosts, and would not compete against those with urban adaptive potential as the agents of cryptococcosis. In the wild environment of the Amazon Forest, the agent of cryptococcosis was isolated at low density (1/148 = 0.67%), possibly due to competition and balance with other microorganisms, whose diversity is inherent in ecologically preserved environment [23].

A clear difference occurs in the distribution of *C. neoformans* and *C. gattii*, with a global prevalence of *C. neoformans* and a higher prevalence of *C. gattii* in the Americas. *C. gattii* was rarely isolated in Europe and Asia [23]. Difference in distribution also occurs with the molecular types. *C. neoformans* VNI prevails worldwide, while VNIV is more common in Europe. *C. gattii* VGI is the most common molecular type [20] worldwide, while *C. gattii* VGII is more common on the American continent.

C. gattii VGI has been isolated from hollow trees and plant debris in Netherlands, Spain and Argentina [12,6,7]. However the number of colonies isolated was significantly smaller than on this study. For the first time VGI was isolated associated with trees in the city of Rio de Janeiro, in a district placed in the south area of the city. This area is covered by species of ficus, pottery trees, munguba and cassia trees. The majority of the hollows observed in the present study were formed in large trunks of *Ficus microcarpa* planted decades ago, allowing the establishment of microbial communities and shelters for wildlife inside their hollows. The high density of VGI colonies found inside them, such as up to 15.700 CFU/g for *C. gattii* and up to 56.250 CFU/g for *C. gattii* VGI co-isolated with *C. neoformans*, point to potential risk of exposure for people and pets that usually frequent those squares. Therefore, tree habitats adapted to urban areas may reach high concentration of propagules of *C. gattii*, constituting microfoci. The hollow trees with *C. gattii* VGI in this study are distributed in clusters, similar to that observed in British Columbia [13].

The four positive trees with *C. gattii* VGI side by side in Marinha do Brasil's square (Fig. 3) may also suggest a dispersal mechanism by insects, rodents, bats and or birds living in the surrounding area. Significantly, the large amount of ants inside and outside the studied hollows called our attention. The possible spread of the agents of cryptococcosis by ants was recently suggested by Jesus et al. [25] who reported the isolation of *C. neoformans* VNI and VNII from the surface of *Odontomachus bauri* ants collected in a hollow tree in Rio de Janeiro.



Fig. 3. The arrows show the four trees arranged side by side, positive for *C. gattii* VGI in Marinha do Brasil square

The only previous report of environmental contamination by *C. gattii* in Rio de Janeiro refers to its isolation from bat excreta collected in the attic of a very old building in the neighbor district of Humaita [8], adjacent to the present study area. This isolate was stored and later on identified as genotype VGI [26]. The present finding of this genotype in hollows of ficus trees in a contiguous area suggests that *C. gattii* VGI circulates or even expands in the southern districts of Rio de Janeiro and that bats may play a role in its dispersion.

The co-isolation of *C. neoformans* VNI and *C. gattii* VGI in a ficus tree was only possible due to the large number of colonies studied and, curiously was detected in the hollow that presented the highest density of *C. gattii*. Thus, if only few colonies are analyzed, possibly this sympatric colonization by different genotypes would not be detected. Co-isolation of *C. gattii* and *C. neoformans* in hollow trees has been formerly reported twice in Brazil: 1) from *Cassia grandis* (VGII and VNI) and *Moquilea tomentosa* (VGII and VNIII) in the city of Teresina, capital of the state of Piauí, Northeast region [26]; 2) *Senna siammea* (VGII and VNI) in the city of Belém, Pará state, North region [27]. It is noteworthy that in these regions the genotype *C. gattii* VGII predominated in the environmental samples studied. Co-isolation of different genotypes have been also reported from other countries: Argentina (VGI and VNI) in *Tipuana tipu* [12]; India (*C. gattii* serotype B and *C. neoformans* serotype A in *Azadirachta indica*, *Cassia fistula*, *Mangifera indica*, *Manilkara hexandra*, *Mimusops elengi*, *Polyalthia longifolia*, *Syzygium cumini* and *Tamarindus indica* [28,29,30], and in one site in Canada (VGI, VGII and VNIII) [13]. Thus, sympatric colonization by different genotypes of *C. gattii* and *C. neoformans* in hollows, or merely decaying wood, appears to be a broader phenomenon. Possible competitive interactions between them and their implications for population structure require further environmental studies.

Cryptococcosis by *C. gattii* in the state of Rio de Janeiro is mainly caused by VGI and VGII, but in most cases it is difficult to determine the state or geographical region where the

infection was acquired due to the great mobility of patients. Although the genotype VGII has not yet been identified in trees in Rio de Janeiro, an autochthonous case of *C. gattii* VGII infection in an infant in the metropolitan area of Rio suggests its local occurrence [19] and a possible expansion of VGII from the North and Northeast of the country, where this molecular type is endemic [15]. The study of environmental sources related to hollow trees in Brazil also shows the predominance of *C. gattii* in the N and NE [15]. However one must consider that cases by *C. gattii* occur in all regions of Brazil and, up to now, they are occasional and less frequent than infections by *C. neoformans* in S and SE. In the country there are no reports of outbreak in humans, however an outbreak in captive psittacine birds caused by *C. gattii* was reported in the state of São Paulo, in the Southeast region [31].

4. CONCLUSION

Predominant occurrence of *C. gattii* VGI in *Ficus microcarpa* tree inhabiting southern districts of Rio de Janeiro has been reported for the first time. Notably, this genotype is also a predominant cause of human infections in this area suggesting an epidemiologic relationship.

Therefore, it is necessary to establish surveillance on the expanding *C. gattii* infections and possible changes in environmental sources in this region of Brazil.

ACKNOWLEDGEMENTS

The authors thank Dr. Mauricio de Andrade Perez for the statistical support. This work was financially supported by the grant E-26/110.486/2007 from "Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ)", Rio de Janeiro, Brazil.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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