First isolation of *Cryptococcus gattii* molecular type VGII and *Cryptococcus neoformans* molecular type VNI from environmental sources in the city of Belém, Pará, Brazil

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Cryptococcus neoformans *and* Cryptococcus gattii *are important agents of meningoencephalitis in humans in the city of Belém. This clinical data suggests that the region may be a highly endemic area for the pathogenic* Cryptococcus *species within the state of Pará (PA), Northern Brazil. Preliminary analysis of 11 environmental samples from the city of Belém showed two positive locations, including a hollow of a kassod tree (*Senna siamea*) colonized simultaneously by* C. gattii *molecular type VGII and* C. neoformans molecular *type VNI, and a birdcage in a commercial aviary positive for* C. neoformans*, molecular type VNI. This is the first evidence of an environmental occurrence of molecular types VNI and VGII in PA.*

Key words: *Cryptococcus neoformans* - *Cryptococcus gattii* - cryptococcosis - Belém - Pará

Cryptococcosis is a life-threatening systemic mycosis affecting healthy and immunocompromised hosts and is globally endemic. It is caused by two species of *Cryptococcus*: *Cryptococcus neoformans* (serotypes A, D, and hybrid AD) and *Cryptococcus gattii* (serotypes B and C), which differ genotypically, phenotypically, in epidemiology, as well as in their geographic distribution and ecologies (Perfect & Casadevall 2002, Kwon Chung & Varma 2006). Mating type in this species is determined by a single genomic locus, with two idiomorphs termed MAT α and MAT a, making this species bipolar. MAT α strains are 30-40 times more prevalent than MAT a strains in most clinical and environmental sampling studies (Kwon-Chung & Bennett 1992). Eight major molecular types have been identified within these two pathogenic species: *C. neoformans* is grouped into the molecular types VNI/AFLP1 (serotype A), VNII/AFLP1A (serotype A), VNIII/AFLP2 (serotype AD) and VNIV/AFLP3 (serotype D); *C. gattii* is grouped into molecular types VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5 and VGIV/AFLP7 (serotypes B and C) (Meyer et al. 1999, Boekhout et al. 2001). These molecular classifications have been used for global epidemiological studies on cryptococcosis.

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Cryptococcal infections are most often acquired through the inhalation of viable propagules from the environment. Although the lungs are the primary sites for the infection, dissemination leads to meningoencephalitis, which is the most frequently diagnosed clinical manifestation in humans. *C. neoformans* has a worldwide distribution, can be found in avian guano and tree hollows, and affects mainly immunocompromised hosts. *C. gattii* distribution prevails in tropical and subtropical regions and the organism is often associated with decomposing wood in the hollows of tropical trees. Several tree species have been found to be colonized by *C. gattii,* including *Eucalyptus camaldulensis,* in Australia and Mexico (Ellis & Pfeiffer 1990, Licea et al. 1996), *Terminalia cattapa,* in Colombia (Callejas et al. 1998), and *Syzygium jambolana, Cassia grandis*, *Senna multijuga*, *Ficus microcarpa*, *Moquilea tomentosa* and *Guettarda acreana,* in Brasil (Lazera et al. 1996, 2000, 2005, Fortes et al. 2001).

C. gattii has the potential to cause life-threatening disease in immunocompetent hosts and is recognized as the main agent of endemic primary cryptococcosis in the Northeast Region of Brazil (Nishikawa et al. 2003). In addition, *C. gattii* has been the agent of one outbreak in captive psittacine birds in São Paulo, Brazil (Raso et al. 2004). *C. gattii* has attracted particular attention as a primary emerging pathogen on Vancouver Island, Canada, where an ongoing large-scale cryptococcosis outbreak in both humans and animals has been caused almost exclusively by *C. gattii* molecular type VGII (Kidd et al. 2004).

In 1999, Corrêa et al. reported a total of 19 cases of cryptococcosis in children in PA and nine of these cases

were caused by *C. gattii* infections. A recent study in the same region showed that *C. gattii* is an endemic primary mycosis affecting HIV-negative hosts, including an unexpectedly high number of children, with most cases caused by molecular type VGII (Santos et al. 2008). Considering that some of these patients were from the Metropolitan area of Belém, the capital of PA, potential environmental sources for cryptococcal infection in this area were investigated in this current study.

The city of Belém (01°27'20"S 48°30'15"W) has approximately 1.280,614 inhabitants. Located at the mouth of the Amazon River, the city has a tropical and humid climate, with a consistently high relative humidity of about 85% (Prefeitura Municipal de Belém 2006).

For a preliminary analysis, sampling was performed in the quarters São Braz and Umarizal, both close to the Tropical Medicine Centre of the Federal University of PA, near the downtown region of the city. Four samples of caged psittacine dried bird excreta in three avian stores were collected. In addition, seven samples of decaying wood material were collected from each tree trunk hollow from trees found along the sidewalk. The trees included four *Senna* sp., one *Senna siamea* tree, one living tree that was not identified, and one trunk of a dead tree. Processing was conducted according to previously published protocols from Lazera et al. (1996).

Positive phenoloxidase colonies were isolated and tested for both thermotolerance at 37ºC and cycloheximide sensitivity. The canavanine-glycine-bromothymol blue medium was used to determine if isolates were *C. gattii* (positive reaction and growth) or *C. neoformans* (negative reaction and no growth). Carbon and nitrogen compound assimilation was performed by the use of the Vitek 32-BioMerieux System (Vitek ICB, bioMeriux, Durham, USA) and the Crypto check kit (Iatron Laboratories, Tokyo, Japan) was used for serotyping of the isolates. After identification, the isolates were stored in Skim Milk medium (DIFCO) at -20ºC.

Genomic DNA was extracted from isolates according to protocols from Ferrer et al. (2001). The mating type was determined using specific PCR primer pairs for mating type α and a, according to Chaturvedi et al. (2000). The α-mating-type-specific primers were 5' -CTTCACTGCCATCTTCACCA-3' and 5' -GACACAAAGGGTCATGCCA-3', While the a-mating-type-specific primers were 5' -CGCCTTCACTGCTACCTTCT-3' and 5' -AACGCAAGAGTAAGTCGGGC-3'. Two type strains, ATCC 28957 (Mat α) and ATCC 28958 (Mat a), were used as positive controls.

Restriction Fragment Length Polymorphism (RFLP) analysis using the *URA5* gene was performed as described by Meyer et al. (2003) using the primers URA5 (5'ATGTCCTCCCAAGCCCTCGACTCCG3') and SJ01 (5'TTAAGACCTCTGAACACCGTACTC 3'). RFLP patterns were assigned visually by comparing them with the patterns obtained from the standard type strains (VNI-VNIV and VGI-VGIV).

One aviary store and one tree were positive for *Cryptococcus*. Five darkbrown colonies were obtained from a sample of bird droppings and all were identified as *C. neoformans* serotype A, MAT α, molecular type VNI. Two dark brown colonies were obtained from decaying wood inside a hollow of a *S. siamea* tree (kassod tree); one colony was identified as *C. neoformans* serotype A, MAT α, molecular type VNI and the other colony was identified as *C. gattii* serotype B, molecular type VGII. Mating type analysis is shown in Fig. 1 and the RFLP profiles are illustrated in Fig. 2.

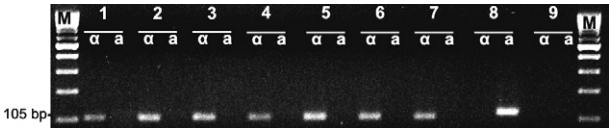

Fig. 1: PCR amplification of environmental isolates with primers MAT α1, MAT α2 and MAT a1, MAT a2. Lanes: M: molecular weight marker: 100 bp DNA ladder; 1: LMM 1082; 2: LMM 1083; 3: LMM 1084; 4: LMM 1088; 5: LMM 1089; 6: LMM 1090; 7: ATCC 28957; 8: ATCC 28958; 9: negative control.

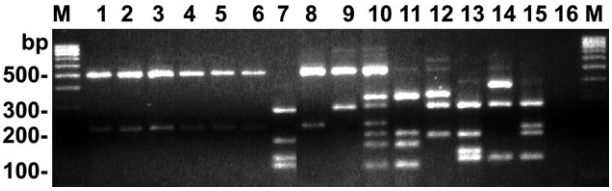

Fig. 2: molecular typing profiles generated via Restriction fragment length polymorphism analysis of URA5 from environmental isolates. Lanes: M: molecular weight marker: 100 bp DNA ladder; 1: LMM 1082; 2: LMM 1083; 3: LMM 1084; 4: LMM 1087; 5: LMM 1088; 6: LMM 1089; 7: LMM 1090; 8: LMM 794; 9: LMM 795; 10: LMM 796; 11: LMM 797; 12: LMM 798; 13: LMM 799; 14: LMM 800; 15: LMM 801; 16: negative control. All samples are molecular types VNI, except Lane 7, which is VGII. Lanes 8-15 are molecular type standard strains (VNI-VNIV and VGI-VGIV).

Few studies on the eco-epidemiology of *C. neoformans* and *C. gattii* have been performed in Northern Brazil. Fortes et al. (2001) demonstrated the occurrence of *C. gattii* in *G. acreana* in a wild area of the Amazon rainforest. In the present study, we report for the first time the isolation of *C. neoformans* and *C. gattii* from environmental sources in the city of Belém, PA. Trilles et al. (2008) analyzed the geographic distribution of *C. neoformans* and *C. gattii* molecular types from isolates within Brazil, and described VNI as the most common molecular type and VGII as the prevailing molecular type in immunocompetent hosts in the North and Northeast Regions of Brazil. Although a large number of isolates were analyzed (n = 443), none were from PA. Recently, Santos et al. (2008) analyzed the agents of 43 cases of cryptococcosis and found that VNI was the most common type in immunocompromised patients, while molecular type VGII was the major cause of endemic pri-

mary mycosis in HIV-negative individuals, including an unexpectedly high number of children in PA.

Kidd et al. (2004) reported *C. gattii* molecular type VGII as the causative agent of the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). In addition, molecular studies of clinical and environmental isolates reported by Escandón et al. (2006) demonstrated the predominance of molecular type VGII within *C. gattii* isolates from Colombia. Moreover, the detection of molecular type VGII in a hollow of a tree in the city of Belém reinforces that this molecular type deserves increased attention in other parts of the Brazilian Amazonia as well as in other South American countries.

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