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Cryptococcus neoformans and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a Southern Brazilian Zoological Garden

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Abstract

Cryptococcus neoformans, a major pathogen in immunocompromised patients, is a ubiquitous free-living fungus that can be isolated from soils, avian excreta and plant material. To further study potential saprophytic sources of this yeast in the Southern Brazilian State Rio Grande do Sul, we analyzed fecal samples from 59 species of captive birds kept in cages at a local Zoological Garden, belonging to 12 different orders. Thirty-eight environmental isolates of *C. neoformans* were obtained only from Psittaciformes (Psittacidae, Cacatuidae and Psittacula). Their variety and serotype were determined, and the genetic structure of the isolates was analyzed by use of the simple repetitive microsatellite specific primer M13 and the minisatellite specific primer $(GACA)_4$ as single primers in the PCR. The varieties were confirmed by pulsed-field gel electrophoresis (PFGE). Thirty-three isolates (87%) were from the var. *grubii*, serotype A, molecular type VNI and five (13%) were *Cryptococcus gattii*, serotype B, molecular type VGI. All the isolates were mating type α. Isolates were screened for some potential virulence factors. Quantitative urease production by the environmental isolates belonging to the *C. gattii* was similar to the values usually obtained for clinical ones.

Key words: avian excreta, cryptococcosis, *Cryptococcus gattii*, *Cryptococcus neoformans*, molecular typing, virulence factors

Introduction

Cryptococcus neoformans, a basidiomycetous yeast, is the etiologic agent for cryptococcosis, a major opportunistic mycosis in patients with AIDS [1–3]. Strains of *C. neoformans* are traditionally divided into five distinct serotypes: A, B, C, D and AD [1]. Due to the fact that strains of different serotypes often exhibit significant divergence at molecular level, three biochemically and genetically distinct varieties have been proposed to accommodate this divergence: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotypes B and C). *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* correspond to the teleomorph *Filobasidiella neoformans* var. *neoformans* and *C. neoformans* var. *gattii* to the teleomorph *F. neoformans* var. *bacillispora* [1, 3–5]. However, sequence analysis of the intergenic spacer (IGS) associated with rDNA [6] and amplified fragment length polymorphism (AFLP) genotyping [7] revealed significant differences in nucleotide composition and a considerable genetic divergence between and

within the former three varieties proposed, indicating that *C. neoformans* var. *grubii* (serotype A) should not be recognized as a separate variety [6]. Based on these studies the authors proposed to divide *C. neoformans* into two separate species, with serotypes A, D, and AD included in the species *C. neoformans* (Sanfelice) Vuillemin, and strains of serotype B and C constituting a new species, *Cryptococcus bacillisporus* Kwon-Chung [6, 7]. More recently a proposal to conserve the name *Cryptococcus gattii* against *C. bacillisporus* was made [8].

The incidence of cryptococcosis caused by *C. neoformans* var. *grubii*/*neoformans* has been increasing steadily because of the AIDS epidemic and the growing use of immunossupressive drugs. Pigeon (*Columba livia*) excreta are considered to be the principal source of these varieties, which explains why the majority of studies have been directed at investigating the fungus in this substrate [1, 9, 10]. However, the frequent isolation of the vars. *grubii* and *neoformans* from excreta of other birds, such as psittacines in zoos, pet shops and private households [11–14], the evidence of zoonotic transmission of the fungus to an immunocompromised patient [15], and some other evidences [16–18], points to the excreta of these birds as possible important environmental reservoirs of this pathogen. In Brazil, *C. gattii* occurs associated with *Eucalyptus* spp. and some other trees and the infection is endemic, especially in the north and northeast of the country being 62.7% of the clinical isolates and 62% of the environmental isolates from serotype B [19]. *C. gattii* was previously associated with deaths of psittacine birds in a breeding aviary in São Paulo, Brazil, and it was also isolated from excreta of these birds. Histological examination of the birds showed cryptococcal cells in the beak, choana, sinus, lungs, air sacs, heart, liver, spleen, kidneys, intestines and central nervous system [14]. In the present report the birds were healthy and did not present incoordination, progressive paralysis, difficulty in flying or superficial lesions.

PCR fingerprinting and other molecular techniques have extended our knowledge of the epidemiology of cryptococcosis over the last few years. PCR fingerprinting has been used to amplify hypervariable repetitive DNA sequences in *C. neoformans*. The pattern generated with the oligonucleotide primer 5′(GACA)$_4$-3′ and the microsatellite specific primer M13 are able to classify *C. neoformans* strains in serotypes A, B/C, or D, showing the sensitivity to detect both inter- and intravarietal differences [9, 10, 20–25]. PCR for *MAT*α and *MAT*a pheromones is being used to determine mating type, ploidy, and variety [26]. Electrophoretic karyotyping (EK) has also been shown to be a useful technique for distinguishing *C. neoformans* isolates. The general differences in chromosome sizes and numbers allow a reasonable prediction of the strain's variety status from its initial karyotype pattern [1, 27, 28].

The present study was motivated by the occurrence of cryptococcosis in the Southern Brazilian State Rio Grande do Sul, and by the fact that excreta of birds other than pigeons have never been analyzed as potential natural reservoirs and sources of the fungus in this state. Our purposes were: (1) verify the presence of *C. neoformans* in the excreta obtained from captive birds at a local Zoo; (2) screen some virulence-associated traits among the isolates; (3) survey the presence of the mating types alleles: α and **a**; (4) apply (GACA)$_4$, M13 PCR fingerprinting and EK to identify the varieties.

Materials and methods

Environmental and animals sampling

Desiccated excreta from 55 cages with 59 species of birds belonging to 12 different orders (Psittaciformes, Piciformes, Galliformes, Falconiformes, Casuariiformes, Strigiformes, Ciconiiformes, Anseriformes, Rheiformes, Struthioniformes, Passeriformes and Columbiformes) were collected in the summer at the Zoological Garden of Sapucaia do Sul, state of Rio Grande do Sul, Brazil, and examined for the presence of *C. neoformans*. The majority of the birds were kept as the only species per cage apart from two of these cages each one with two and three species of birds. Imported or quarantine animals were never considered. The examined birds were healthy with a residency of at least 3 months, and none of them had been treated with drugs for at least 1 week before sampling. Air contamination was verified with birdseed agar plates supplemented with ampicillin (150 μg ml^{-1}), chloramphenicol (150 μg ml^{-1}) and biphenyl

Table 1. C. neoformans isolates from Psittaciformes studied by environmental sources, variety (serotypes) and molecular type as identified by PCR fingerprinting with the primers $(GACA)_4$ and M13

Bird scientific name (common name)	Isolate	Identification	Serotype	Molecular type
Aratinga leucophthalmus (White-eyed parakeet)	AZ01; AZ02; AZ03	*C. n.* var. *grubii*	A	VNI
Pionus maximiliani (Scaly-headed parrot)	AZ04; AZ05; AZ06; AZ07	*C. n.* var. *grubii*	A	VNI
Nymphicus hollandicus (Cockatiel)	AZ 08	*C. n.* var. *grubii*	A	VNI
Psittacula eupatria (Alexandrine parakeet)	AZ15; AZ16; AZ17	*C. n.* var. *grubii*	A	VNI
Nandayus nenday (Nanday parakeet)	AZ18; AZ19; AZ20; AZ21; AZ22; AZ23	*C. n.* var. *grubii*	A	VNI
Aratinga aurea (Peach-fronted conure)	AZ24; AZ25; AZ28	*C. n.* var. *grubii*	A	VNI
Aratinga aurea (Peach-fronted conure)	AZ26; AZ27	*C. gattii*	B	VGI
Aratinga jandaya (Jandaya parakeet)	AZ09; AZ10; AZ11	*C. n.* var. *grubii*	A	VNI
Amazona festiva (Festive parrot)	AZ29; AZ30; AZ31; AZ33; AZ34; AZ35; AZ36; AZ37	*C. n.* var. *grubii*	A	VNI
Amazona festiva (Festive parrot)	AZ32	*C. gattii*	B	VGI
Amazona rhodocorytha (Red-brown amazon parrot)	AZ12	*C. n.* var. *grubii*	A	VNI
Amazona farinosa (Mealy parrot)	AZ13; AZ14	*C. gattii*	B	VGI
Amazona farinosa (Mealy parrot)	AZ38	*C. n.* var. *grubii*	A	VNI

(0.1%) [9], which where left open during 15 min inside the cages and also outside by 0.5 m of the cages. These plates where incubated for 14 days a 30 °C with daily observation. Samples from hollows of two eucalypt trees situated near the cages were also collected as previously described [4].

Fecal specimens sampling and processing

Approximately 20 g of excreta were taken from the cage floors using sterile spatulas, put into sterile plastic containers and processed on the same day. Approximately 1 g of the avian excreta was diluted in 9 ml of sterile saline solution (0.9% NaCl), vortexed for 10 min, and allowed to settle for 1 h. Aliquots of 10^{-2} and 10^{-3} dilutions were inoculated in duplicate onto birdseed agar plates supplemented with ampicillin (150 μg ml^{-1}), chloramphenicol (150 μg ml^{-1}) and biphenyl (0.1%) [9]. The plates were incubated at 30 °C and examined each day for the presence of colonies showing the brown color effect (BCE). The colonies with BCE were enumerated after 72–96 h of growth and the plates were observed for a period up to 14 days. Representative BCE colonies were streaked twice in birdseed agar, and the isolates were maintained in Sabouraud-dextrose agar slants until identification and characterization [9].

Identification and maintenance of the isolates

The identification of the isolates included microscopic analysis of India ink preparations, thermotolerance at 37 °C in Sabouraud dextrose agar, cycloheximide sensitivity (0.1%), qualitative urease activity [29], NaCl sensitivity, nitrate and carbon assimilation tests [9, 30]. The canavanine-glycine-bromothymol blue (CGB) medium was used to determine the variety of the isolates [31]. Serotyping was performed using the Crypto Check kit (Iatron Labs, Tokyo, Japan) according to the manufacturer's instructions. After identification,

the *C. neoformans* isolates were maintained on Sabouraud dextrose agar at 4 °C and in glycerol stocks at −20 °C.

Assay of virulence factors

Extracellular phospholipase production was assayed in Sabouraud dextrose agar with 1 M sodium chloride, 5 mM calcium chloride and 8% sterile egg yolk [10]. The diameter of the zone of precipitation around the colonies was measured after 7 days incubation at 30 °C. The index of phospholipase activity (P_z) was a ratio between the colony diameter and the total diameter of the colony plus precipitation zone. A P_z value of 1.0 indicated that the test sample was phospholipase negative. Urease activity was determined with a phenol–hypochlorite reaction, according to Weatherburn [32].

Determination of mating type by PCR

DNA was extracted as previously described [10]. Two PCR primer pairs, specific for mating type α and a, were used, according to Chaturvedi et al. [26]. The α-mating-type-specific 5′ -primer was 5′-CTTCACTGCCATCTTCACCA-3′ (*MAT*α1) and the 3′ -primer was 5′-GACACAAAGGGTCA TGCCA-3′ (*MAT*α2). The a-mating-type-specific 5′-primer was 5′-CGCCTTCACTGCTACCTTC T-3′ (*MAT*a1) and the 3′ -primer was 5′-AACGCA AGAGTAAGTCGGGC-3′ (*M AT*a2). Amplification reactions were performed in a volume of 25 μl. The PCR amplicons were electrophoresed on 2% agarose gels in 1× Tris-borate-EDTA (TBE) buffer at 80 V for 90 min and then stained in a solution of 0.5 μg ml^{-1} ethidium bromide.

PCR fingerprinting

Oligonucleotides of the minisatellite specific core sequence of the wild-type phage M13 (5′-GAG-GGTGGCGGTTCT-3′) and of the microsatellite specific sequence $(GACA)_4$ were used as single primers in the PCR [21]. Amplification products were separated by electrophoresis in 1.4% agarose gels in 1× TBE buffer stained with ethidium bromide at 0.5 μg ml^{-1} for 7 h at 60 V and visualized under UV light. PCR fingerprinting types (VNI–VNIV and VGI–VGIV) were assigned according to the molecular standards provided by the Molecular Mycology Laboratory in Sydney, Australia.

Electrophoretic karyotyping

Karyotype analysis was done by contour-clamped homogeneous electrophoresis (CHEF DR-II). *C. neoformans* chromosomal DNA plugs were prepared using a modification of existing protocols [27, 28]. For EK, the plugs were inserted into a 1% pulsed-field-certified agarose (Bio-Rad) gel (12 by 14 cm), and electrophoresis was performed in a CHEF DR-II variable-angle pulsed-field electrophoresis system (Bio-Rad) in 0.5× TBE buffer (1× TBE is 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA pH 8.3) at 14 °C. Electrophoretic conditions were as follows: pulse intervals of 60 to 120 s for 39 h at 6 V/cm. The gels were stained with ethidium bromide and photographed. A *Saccharomyces cerevisiae* chromosomal DNA size standard was inserted in each gel as a molecular weight standard.

Results

Isolation and identification of C. neoformans

C. neoformans was isolated from 10 out of 55 bird excreta samples (18.18%). Positive samples were only from excreta of psittacine birds maintained as one species per cage located in the same area. Thirty-eight isolates were obtained. The positive psittacines fecal samples were further screened during the winter but isolates of *C. neoformans* were not obtained. Microscopic examination of fresh isolates in birdseed agar with India ink preparations was used to observe the presence of capsule in the isolates. Thirty-three out of 38 isolates exhibited non-capsulated cells or cells with a tinny capsule, associated with smooth and dry colony morphologies on birdseed agar. Five isolates presented medium to large capsules, correlating with a mucous and brilliant colony morphologies on the same medium. All isolates were nitrate-negative, urease-positive and their growth was inhibited in presence of cycloheximide 0.1% and NaCl 16%. All isolates were *C. neoformans* according to the assimilation pattern of

19 carbon compounds. Glucose, galactose, sorbose, sucrose, trehalose, raffinose, maltose, D-ribose, L-rhamnose, D-xylose, myo-inositol and D-mannitol were assimilated by all isolates, whereas lactose, melibiose, *N*-acetil-glucosamine, L-arabinose and glycerol were not. Assimilation of cellobiose, and glucosamine was variable. Thirty-three isolates (87%) were *C. neoformans* var. *grubii*/*neoformans* according to biotyping in CGB agar and 5 (13%), as *C. gattii*. Serotypes were confirmed with commercial monoclonal antibodies (Iatron Labs, Tokyo, Japan). Air sampling around the cages and samples collected from eucalypt trees were negative for the presence of *C. neoformans*.

Putative virulence factors

Extracellular phospholipase production was readily observed as distinct, cream zones of precipitation around the colonies in 19 (50%) out of 38 isolates. The remaining 19 isolates failed to induce a precipitate after 7 days of incubation at 30 °C. The environmental isolates exhibited P_z values within a range of 0.69–0.94, with an average of 0.83 (data not shown). Only one out of five isolates of *C. gattii* presented phospholipase activity. The quantitative urease results are shown at Figure 1. The five isolates identified as *C. gattii* had a higher urease activity than the other isolates.

Determination of mating type by PCR

Two type strains, ATCC 28957 (serotype D, *MAT*α) and ATCC 28958 (serotype D, *MAT*a) and one reference strain (ATCC 32045 *MAT*α/a) [10] were used as positive control. A 101-bp *MAT*α fragment and a 117-bp *MAT*a fragment were amplified from the corresponding controls. All the isolates belonged to mating type α (data not shown).

PCR Fingerprinting and Electrophoretic karyotyping

The microsatellite specific primer M13 and the minisatellite specific primer $(GACA)_4$ were used to amplify DNA polymorphisms within the genome of the 38 isolates. Representative gels can be seen in Figure 2. PCR-fingerprinting with the primers $(GACA)_4$ and M13 separated the *C. neoformans* isolates into two molecular types: VNI representing serotype A, and VGI representing *C. gattii*, serotype B. The most common molecular type was VNI (serotype A, var. *grubii*), which was present in 33 (87%) isolates. Five (13%) isolates belonged to VGI (serotype B, *C. gattii*).

Data regarding EK on environmental samples of *C. neoformans* are limited. Among the three representative isolates from *C. neoformans* var. *grubii* and from *C. gattii* used for EK, four unique profiles were noted upon visual inspection by using a one band difference as the discrimination criteria (Figure 3). *C. gattii* isolates had indistinguishable patterns showing small chromosomes (less than 700 kb) seen frequently in this specie [33]. However, all *C. neoformans* var. *grubii* isolates had different karyotypes as also observed by Perfect et al. [34] for environmental isolates found in soil or bird guano in North Carolina and Ohio.

Discussion

The isolation of *C. neoformans* in nature took place for the first time from peach juice in 1895, and since the early 1950s, researchers have known that this fungus is associated with avian excreta [1, 35]. Comparative studies have suggested that excreta of some species of birds may be more likely to be contaminated with the fungus than others, being a good nutrient for growth and survival for this pathogen, and may therefore, represent a reservoir and possible source of infection [1, 17]. Although in birds clinical cryptococcosis is rare, an outbreak was described for psittacine in the families Loriidae and Psittacidae in Brazil. The birds died of disseminated cryptococcosis caused by *C. gattii* [14].

It has been difficult to link human infection to bird excreta exposure; however there are some studies that strongly suggest zoonotic transmission [15, 18]. *C. neoformans* has been already isolated from excreta of captive birds in zoos [11, 16, 36, 37] and the control of the habitats of the fungus in zoological gardens and similar establishments is considered a necessity to prevent exposure of susceptible persons [12].

The 38 isolates obtained in this work from excreta of captive birds were all identified as *C. neoformans*, with 33 identified as *C. neoformans* var. *grubii* and 5 isolates as *C. gattii*. These five isolates were confirmed as *C. gattii* by the characteristic

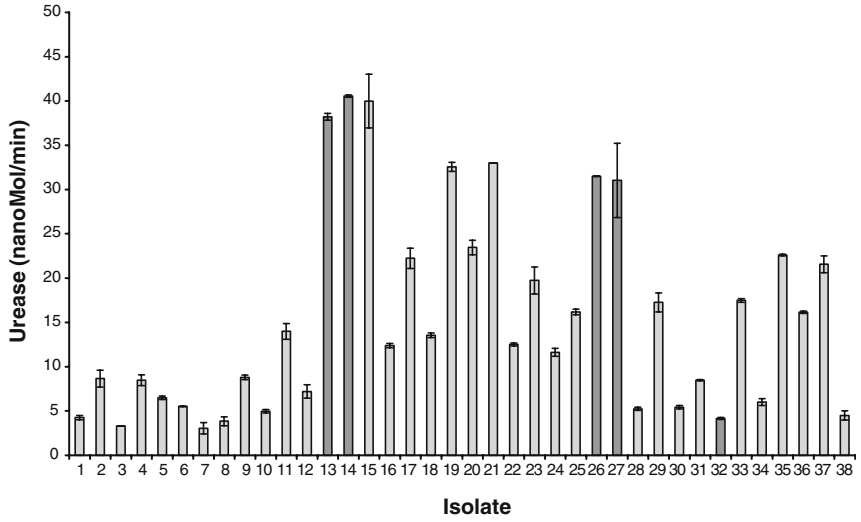

Figure 1. Urease activity of environmental samples of *C. neoformans* var. *grubii* (light shading) and *C. gattii* (dark shading) obtained from the Zoological garden. For other isolate characteristics see Table 1.

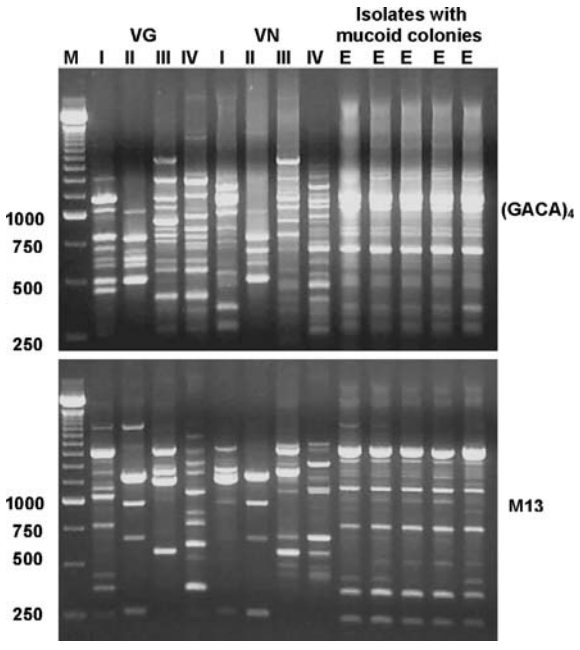

Figure 2. Representative gels of PCR fingerprinting patterns from some of the environmental *C. neoformans* isolates obtained from the Zoological garden. Upper panel primer $(GACA)_4$ and lower panel primer M13. VG and VN molecular types patterns of reference samples. M: molecular size marker, 250 bp ladder. E: environmental samples, AZ13, AZ14, AZ26, AZ27 and AZ32 respectively, all *C. gattii.* For other isolate characteristics see Table 1. Reference strains are: VGI, WM179; VGII, WM178; VGIII, WM161; VGIV, WM779; VNI, WM148, VNII, WM626; VNIII, WM628; VNIV, WM629.

color at the CGB medium, serology (serotype B), $(GACA)_4$ and M13 PCR-fingerprinting (molecular type VGI), and EK. The presence of large capsules and mucoid/brilliant colonies in the birdseed agar corresponds to the characteristic morphology of *C. gattii* [1]. *C. neoformans* was not recovered from eucalypts trees near the cages or from the birdseed agar plates left open inside the cages ruling out the possibility that the fungus was in the overall environmental rather than specifically in the bird's excreta. Reports of cryptococcosis by *C. gattii* in Brazil are sparse. The association of this variety with alternative ecological niches opens the possibility that the cryptococcosis caused by serotype B isolates in the South region of Brazil may be greater than previously thought [19].

The majority of the 38 isolates were serotype A and molecular type VNI, what is consistent with the fact that *C. neoformans* var. *grubii* is prevalent among environmental sources [20, 21]. The isolates were all obtained from excreta of psitaccines, and this material is reported in the literature as a good nutrient for *C. neoformans* growth [1]. However, the incidence of cryptococcal infection due to exposure to pet birds, such as psittacines, is unknown [1, 17]. Recently, Lagrou et al. [18] reported a case of cryptococcal meningitis in an immunocompetent patient with exposure to a pet magpie (Corvidae).

All 38 isolates analyzed were mating type α which is in accordance with the reports that

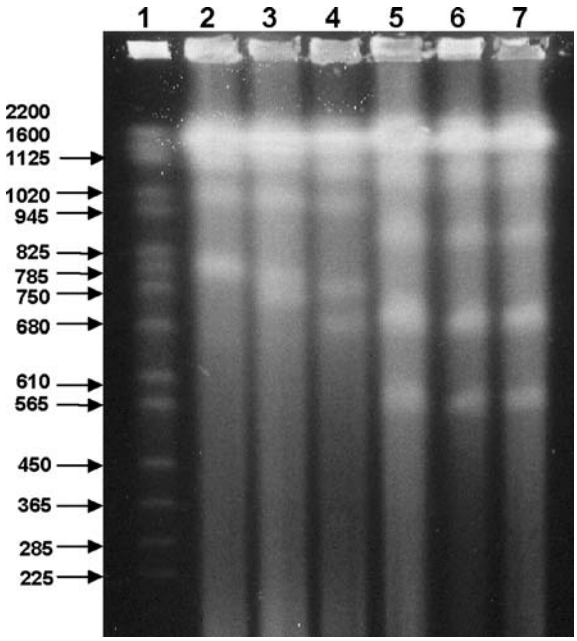

Figure 3. Electrophoretic karyotypes of environmental samples of *C. neoformans* var. *grubii* and *C. gattii* obtained from the Zoological garden. Lanes are: 1, *S. cerevisiae* DNA markers; 2, 3, and 4 correspond to samples AZ06, AZ10 and AZ12 respectively, all var. *grubii*; 5, 6 and 7 correspond to samples AZ13, AZ26 and AZ32 respectively, all *C. gattii*. Numbers in the left indicate molecular sizes in Kb. For other isolate characteristics see Table 1.

mating type α strains are 30- to 40-fold more prevalent than those of the *MAT*a among clinical as well as environmental isolates [10].

In spite of the very similar mean pH of positive and negative samples (7.3 and 7.6, respectively), pH cannot be ruled out as one of the factors that could influence *C. neoformans* survival in the fecal samples. In general, birds with a diet based on citric fruits had excreta with acidic pH (4.0–5.0), and birds with a meat-based diet had excreta with alkaline pH (8.0–9.0). As already known that alkaline pH inhibits fungal growth, this could partially explain the absence of this fungus in the excreta of some bird species.

Analysis of some putative virulence factors of the environmental isolates may suggest the infection potential of these strains. Phospholipase activity was detected in 50% of the isolates, and its P_z value is in accordance with earlier findings for environmental strains [38, 39]. Clinical isolates usually have a higher phospholipase activity, what is known to be important for the virulence of the pathogen [40]. Phospholipase P_z value was similar among the excreta belonging to both varieties. Urease activity was higher in the isolates belonging to *C. gattii* and was comparable to previous results obtained for clinical isolates in our laboratory [10]. The importance of urease activity for the pathogenesis of the fungus is still under debate, but mice infected with urease-negative mutants of *C. neoformans* had a higher percentage of survival than those infected with urease-positive controls [41].

The *C. neoformans* var. *grubii* isolates were all from molecular type VNI, which is the most prevalent worldwide among clinical and environmental strains [10, 20, 21]. The *C. gattii* strains belonged to molecular type VGI. Casali et al. [10] reported that all *C. gattii* analyzed from clinical samples of the state of Rio Grande do Sul were molecular type VGIII. However, the number of clinical isolates of this variety genotyped in that study is small and possibly non-representative of the bulk of isolates. Thus, it is not possible to affirm that the molecular type VGI will not infect people in our local environment. As such, isolation of this molecular type with an indistinguishable karyotyping suggesting the occurrence of a clonal event in the environmental should raise some concerns. Moreover, this clonal event is even more particularly important given the ongoing outbreak in Vancouver Island, BC, Canada [25]. It is clear that there is much to learn about the ecology and epidemiology of this primary pathogen and the importance of continued surveillance in the environment not only in Brazil but worldwide.

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