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Cryptococcus neoformans Isolated from Passerine and Psittacine Bird Excreta in the State of Paraná, Brazil

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Abstract *Cryptococcus neoformans* is an opportunistic basidiomycete yeast that causes life-threatening infections as meningoencephalitis primarily in immunocompromised hosts, generally associated with AIDS. The source of this organism is mainly pigeon excreta; however, other avian species' excreta are implicated as a source of this yeast. The occurrence of *C. neoformans* and *Cryptococcus gattii* in bird excreta in the state of Paraná in Brazil was determined in this study. A total of 141 samples of Passerine and Psittacine excreta from captive birds were collected. Additionally, 25 clinical samples from Hospital de Clínicas, in the state of Paraná were also analyzed. The determination of molecular and mating type of the isolates was performed by PCR fingerprinting, multiplex PCR, and mating type PCR. *Cryptococcus neoformans* var. *grubii* (VNI) was isolated from 36 (25.53%) of Passerine and Psittacine excreta samples. Almost all clinical samples, except one (*C. gattii* VGI), were classified as *C. neoformans* var. *grubii* (VNI). All environmental and clinical isolates were mating type α. These findings reinforce that, besides pigeon excreta, the excreta of these birds can also be a reservoir of *C. neoformans* in domestic and public environments and is of zoonotic importance to immunocompromised patients.

Keywords Avian excreta · *Cryptococcus neoformans* · *Cryptococcus gattii* · Passerines · Psittacines

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Introduction

Cryptococcosis, an opportunistic disease commonly associated with meningoencephalitis in humans, is caused by the yeast *Cryptococcus neoformans* (=*C. neoformans* and *C. gattii*), and is generally associated with immunocompromised conditions such as in patients with AIDS, post-organ transplant surgery, or hematologic malignancies [1–3]. Since the mid-1980s the incidence of cryptococcosis has increased, which may be due to both the growing population of AIDS patients and the use of immunosuppressive drugs. Currently, this opportunistic disease is one of the most common life-threatening mycosis in the world [2]. In Brazil, according to official data, cryptococcosis represents 6% of all infections in AIDS patients [4].

The species complex comprises two species and five serotypes. *Cryptococcus neoformans*, the first species, includes two varieties *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) and a hybrid between the two varieties (serotype AD) [1, 5, 6]. Know-Chung et al. [7] proposed to reclassify the variety *gattii* [8] as the second species of the complex (*C. gattii* serotypes B and C) based on the differences in its morphology, antigenic structure, virulence profile, epidemiology, ecology, and geographic distribution. The mechanisms underlying differences in host specificity and geographical distribution remain unknown. Although the geographic distribution of serotypes and varieties was, until recently, considered to be regionally specific, the variety *grubii* has been recovered from most of the environmental and clinical sources already reported in the world, which mostly corresponds to clinical isolates from AIDS patients [6, 9].

Knowledge of mating type is important for understanding the ecology and virulence of this fungus [10]. Both previously mentioned species are haploid and heterothallic and have a bipolar mating system with single mating type loci and two alleles: MATa and MATα. The MATα strains have been shown to be more prevalent in clinical and environmental isolates and also more virulent than MATa [2].

Cryptococcus neoformans is primarily associated with nests and soils containing avian droppings, especially those of pigeons [11, 12]. Despite the fact that both *Cryptococcus* species, *C. neofornans* and *C. gattii*, are capable of growth on pigeon guano, only *C. neoformans* exhibit prolific mating, completing its life cycle. Then, bird guano may represent the ecological niche for *C. neoformans* [13]. Studies reported that captive birds can promote the contamination of dwellings and public areas by *Cryptococcus* species [14–16]. Cryptococcosis is acquired from the inhalation of airborne propagules from the environment, and is possible because the size of the yeast is compatible with alveolar deposition [1]. Reports have been published describing the development of cryptococcosis in people after exposure to birds or avian excreta, illustrating that human infection can result from these sources [17, 18]. Therefore, Passerine and Psittacine birds have the potential to disseminate *C. neoformans* and other zoonotic agents because both are popular as pets in Brazil.

Because of the high concentration of *C. neoformans* in bird excreta, the high density of birds in urban areas and the severity of cryptococcosis in humans, there is a considerable interest in the understanding of the relationship among birds, their excreta, and human cryptococcosis [1]. The saprobiotic source of *C. neoformans* has been researched in various parts of the world, but many questions concerning the ecology of the fungus and the epidemiology of cryptococcosis remain unresolved. Extensive environmental surveillance of *C. neoformans* within Brazil is necessary to determine the potential environmental reservoirs of each of the molecular types involved in the infections.

The epidemiological knowledge of cryptococcosis is important to understand the pathogenesis of infection and the risk factors associated with the disease. Although there have been few reports on the epidemiology and ecology of *C. neoformans* in Paraná State, Brazil, 101 new cases of cryptococcosis were identified from January 2000 to December 2005, at Hospital de Clínicas (Curitiba, Paraná, Brazil). The incidence of cryptococcosis indicates the presence of infective propagules in the urban area of Curitiba and its surrounding areas, where the yeast must be encountering favorable environmental conditions for development. An understanding of the epidemiology of cryptococcosis may provide a rational framework for the design of prevention guidelines and more effective therapies.

The purposes of this study were: (1) check the existence of *C. neoformans* and *C. gattii* in captive bird excreta in the state of Paraná, Brazil; (2) differentiate *C. neoformans* and *C. gattii* by multiplex PCR; (3) apply M13 and $(GACA)_4$ PCR fingerprinting to understand the molecular epidemiology of clinical and environmental isolates; and (4) detect the predominant mating type in isolates from clinical and environmental samples.

Materials and Methods

Environmental Samples and Fungus Isolation

From October 2004 to November 2005, 141 environmental samples of Passerine and Psittacine excreta (108 from Passerine, 32 from Psittacine birds, and one from a cage containing both order of birds) were

collected from metal fencing, perches, and cage floors in sterile plastic bags using a sterile wooden spatula. Desiccated and weathered excreta were sampled whenever this kind of sample was available. Of these, 19 excreta samples were from captive birds in pet shops located in the city of Curitiba, Paraná, Brazil, and 122 samples were from birds received in Centro de Triagem de Animais Silvestres (CETAS) PUCPR/IBAMA, located in a neighboring city, Tijucas do Sul, Paraná State, Brazil. The birds from CETAS were mostly pet birds from illegal traffic. The majority was confiscated in metropolitan Curitiba and the eastern Atlantic coast with some having come from other parts of the state.

For *Cryptococcus* isolation, 10 g of weathered bird excreta was added to 40 ml of sterile saline solution. The samples were mixed for 2 min, allowed to sit for 10 min, and then 8 ml of the supernatant was resuspended with 2 ml of sterile saline solution containing penicillin (4.5 mg/l) and streptomycin (10 mg/l). Aliquots of 100 µl were spread onto birdseed agar plates (*Guizotia abyssinica*) and incubated at 30°C for up to 7 days, although positive samples were invariably apparent after 48–72 h. The resulting dark brown colonies suggestive of *C. neoformans* were then subcultured on Sabouraud's peptone dextrose agar plates.

The isolates were identified to the species level based on their morphological and biochemical characteristics [2]. Samples for microscopic examination were prepared using India ink or lactophenol cotton-blue stains. All environmental isolates were identified as *C. neoformans* and/or *C. gattii* by standard biochemical methods: ability to produce melanin in birdseed agar, cycloheximide sensitivity in Sabouraud dextrose agar, cycloheximide sensitivity (0.1%), thermotolerance at 37°C, urease production, and carbon and nitrogen assimilation profiles. Each isolate was represented by one *Cryptococcus* colony obtained in birdseed plate [19].

Clinical Isolates

Twenty-five clinical isolates were obtained through the Medical Mycology Laboratory, Hospital de Clínicas, Universidade Federal do Paraná (Curitiba, Paraná, Brazil), in the period from May 2004 to December 2005. The clinical strains were isolated from human patients before the initiation of antifungal therapy. Patients with relapsed cryptococcal infection were excluded. All patients that had isolates of *C. neoformans* had their medical records reviewed to obtain information about their cryptococcosis episodes. The isolates were obtained by routine medical microbiological procedures. These strains were identified by individual and colonial morphology, conventional biochemical test or using YBC Card Vitek System (BioMériex, Marcy l'Etoile, France) and CGB test to determine the species.

Determination of *Cryptococcus* Species

The species (*C. neoformans* or *C. gattii*) of each isolate was determined by the L-canavanine-glycine-bromothymol blue (CGB) medium [20] and multiplex PCR with primers CNa-70S (5′-ATTGCGTCCACCAAGGAGCTC-3′) and CNa-70A (5′-ATTGCGTC CATGTTACGTGGC-3′) for *C. neoformans*; and CNb-49S (5′-ATTGCGTCCAAGGTGTTGTTG-3′), and CNb-49A (5′-ATTGCGTCCATCCAACCGTTATC-3′) for *C. gattii* [21]. Cultures were grown on Saboraud's peptone dextrose agar at 30°C for 48 h prior to DNA extraction. The method used to extract DNA was an adaptation of one already described [22]. PCR were carried out in a mixture of 25 µl containing 10–20 ng of DNA; reaction buffer (10 mM Tris–HCl pH 8.3; 50 mM KCl; 2.3 mm $MgCl_2$); 200 µM of deoxynucleotide triphosphates; 25 pmol of each primer; and 1 U *Taq* DNA polymerase. An initial denaturation at 94°C for 8 min was followed by 35 cycles at 94°C for 1 min, annealing at 65°C for 1 min and elongation at 72°C for 2 min, and a final elongation at 72°C for 8 min. The controls consisted of standard strains: *C. neoformans* (WM 148, serotype A) and *C. gattii* (WM 161, serotype B) [9]. After identification, the isolates were maintained in glycerol stocks. Yeast cells were grown on Sabouraud's agar slants for 48 h and loopful of cells were suspended in 1 or 2 ml of 15% glycerol and 20% skim milk and frozen at −70°C.

PCR Fingerprinting and Mating Type

PCR fingerprinting was performed with the minisatellite-specific core sequence of the wild-type phage M13 (5′-GAGGGTGGCGGTTCT-3′) and of the microsatellite-specific sequence $(GACA)_4$ as single primers in the PCR, according to Meyer et al. [9].

The MATα and MATa were determined according to Chaturvedi et al. [23]. Data were analyzed with SPSS 13.1, using the unweighted pair group method using arithmetic mean (UPGMA) analysis with Dice coefficient and fuzzy logic option. Statistical significance of the clusters was tested by cophenetic correlation and bootstrap analyses.

Statistical Analysis

The non-parametric χ^2 test was used to compare frequencies of positive results according to avian orders (Passerine and Psittacine birds), sample places (Curitiba aviary or CETAS), individual or collective cages and also to compare clinical characteristics from the human samples. *P*-values less than 0.05 were deemed significant.

Results and Discussion

Cryptococcus neoformans was isolated from 34 (24.11%) Passerine samples and two (1.42%) Psittacine samples, with an overall occurrence of 25.53% (Table 1). Despite the fact that pigeon droppings are commonly documented as the major source of *C. neoformans* in nature, the yeast has also been isolated in high rate from droppings of caged birds including both Passerine and Psittacine birds [12, 14]. *Cryptococcus neoformans* is generally isolated from avian droppings, whereas *C. gattii* is not typically isolated [13].

The percentage of caged bird excreta containing *C. neoformans* in our study was higher when compared to those with recent environmental sources associated with birds in other countries [12, 24], but similar to available data from other Brazilian studies [14–16]. Differences in methodology limited the comparison of our results with theirs, but the presence of *C. neoformans* probably depends on avian habitat and climatic aspects occurring in distinct geographical areas.

The number of positive samples was significantly higher for Passerine than for Psittacine birds ($P \leq 0.01$). Canary (*Sicalis flaveola*) excreta had a large number of positive samples but not significantly higher. Sample number 04 (Table 1) was the only one where pigeons were housed with Passerine birds. The number of cage inhabitants and/or species did not significantly influence the presence or absence of yeast.

The uneven number of samples collected from Passerine and Psittacine birds may explain the differences in number of positive samples from each order. The number of samples collected from each species was influenced by the number and types of birds available at CETAS and the availability of pet birds in pet shops from Curitiba, Paraná, Brazil. The composition of the excreta, endosaprobiotic cycle, biochemical adaptations of the yeast, co-infection with free-living amoebae, and bacteria that compete with *C. neoformans* in fecal matter, as well as sun light exposure, diets, reproductive and sanitary habits are other variables that possibly influenced the outcome of this investigation [1, 14]. Furthermore, the distribution of yeasts in the normal digestive tract [25] as well as diets with alkaline pH may produce a lower recuperation of the fungi in excreta [16]. Some avian excreta offer better conditions for the survival and multiplication of *C. neoformans* [1, 13, 14], probably because of carbon sources as glucose in some avian guanos [13].

Most samples collected from Psittacine birds were wet or fresh at the time of collection, and this might have contributed to the failure in the isolation of the yeast [2]. It is well known that wet droppings undergo bacterial decomposition, causing a strong alkalinization of the substrate and growth inhibition of *C. neoformans*. Dry excrement is a favorable substrate because it has fewer viable bacteria and less competition [12], and thus old desiccated excreta is more likely to harbor high numbers of *C. neoformans* [3]. The husbandry conditions for Psittacine birds in captivity are usually better than for Passerine birds, because Psittacines are more often considered to be true pets by the general population and therefore are housed in closer proximity to human being living spaces.

A variety of other fungi grew on the bird seed agar in addition to colonies of *C. neoformans*, but these were not classified further (data not shown). Presence or absence of competing organisms, such as fast-growing filamentous fungi, may be an important factor in whether or not *C. neoformans* was isolated from any given sample [12].

The high contamination rates observed in the cages from CETAS can be related to the high density of cages, daily accumulation of excreta, overpopulated cages, insufficient ventilation, and large amount

Table 1 *Cryptococcus neoformans* isolates from Passerine and Psittacine birds in Paraná state, Brazil

Sample code	Individuals per cage	Species of birds	Identification	Molecular/mating type
02	01	*Sporophila* sp. (PA)	*Cn* var. *grubii*	VNI/α
04	10	*Columbina talpacoti* (CO)	*Cn* var. *grubii*	VNI/α
		Coryphospingus cucullatus (PA)		
		Molothrus bonariensis (PA)		
		Zenaida auriculata (CO)		
		Geopelia cuneata (CO)		
05	01	*Myiopsitta monachus* (PS)	*Cn* var. *grubii*	VNI/α
06	01	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
07	01	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
08	01	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
09	01	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
11	11	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
19	02	*Zonotrichia capensis* (PA)	*Cn* var. *grubii*	VNI/α
		Sicalis flaveola (PA)		
20	01	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
30	01	*Zonotrichia capensis* (PA)	*Cn* var. *grubii*	VNI/α
34	02	*Sporophila caerulescens* (PA)	*Cn* var. *grubii*	VNI/α
35	01	*Sporophila caerulescens* (PA)	*Cn* var. *grubii*	VNI/α
37	01	*Serinus canarius* (PA)	*Cn* var. *grubii*	VNI/α
44	01	*Oryzoborus angolensis* (PA)	*Cn* var. *grubii*	VNI/α
54	04	*Sporophila frontalis* (PA)	*Cn* var. *grubii*	VNI/α
65	01	*Zonotrichia capensis* (PA)	*Cn* var. *grubii*	VNI/α
97	02	*Cardulelis magellanicus* (PA)	*Cn* var. *grubii*	VNI/α
118	01	*Melopsittacus undulatus* (PS)	*Cn* var. *grubii*	VNI/α
133	01	*Saltator similis* (PA)	*Cn* var. *grubii*	VNI/α
134	01	*Platycichla flavipes* (PA)	*Cn* var. *grubii*	VNI/α
136	01	*Platycichla flavipes* (PA)	*Cn* var. *grubii*	VNI/α
137	01	*Turdus albicollis* (PA)	*Cn* var. *grubii*	VNI/α
138	01	*Platycichla flavipes* (PA)	*Cn* var. *grubii*	VNI/α
141	01	*Passerina brissonii* (PA)	*Cn* var. *grubii*	VNI/α
142	01	*Paroaria coronata* (PA)	*Cn* var. *grubii*	VNI/α
143	01	*Turdus albicolis* (PA)	*Cn* var. *grubii*	VNI/α
144	01	*Paroaria coronata* (PA)	*Cn* var. *grubii*	VNI/α
147	01	*Turdus rufiventris* (PA)	*Cn* var. *grubii*	VNI/α
148	01	*Turdus albicolis* (PA)	*Cn* var. *grubii*	VNI/α
149	06	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
		Sporophila frontalis (PA)		
		Zonotrichia capensis (PA)		
150	06	*Sicalis flaveola* (PA)	*Cn* var. *grubii*	VNI/α
		Sporophila caerulensis (PA)		
		Oryzoborus angolensis (PA)		
151	02	*Gnorimopsar chopi* (PA)	*Cn* var. *grubii*	VNI/α

Table 1 continued

Sample code	Individuals per cage	Species of birds	Identification	Molecular/ mating type
157	04	*Gnorimopsar chopi* (PA)	*Cn* var. *grubii*	VNI/α
		Molothrus banariensis (PA)		
160	03	*Sporophila frontalis* (PA)	*Cn* var. *grubii*	VNI/α
		Sporophila falcirostris (PA)		
165	02	*Sporophila caerulensis* (PA)	*Cn* var. *grubii*	VNI/α

PA, Passerine bird; PS, Psittacine bird; CO, Columbiform bird; *Cn*, *Cryptococcus neoformans*

of seeds, which can serve as a substrate for the growth of the fungi [15]. It is likely that the low rate of isolation of the fungus from the pet aviaries in the current study is due to the low quantity of bird excrement available, because of the frequent cleaning and disinfecting of the cages. The only positive samples obtained from aviaries were from the pet shop that appeared to have the worst sanitary conditions.

Despite the prevalence of this yeast in the avian excreta, the source of *C. neoformans* growth in excreta is not yet clear. We have previously tried to isolate *C. neoformans* and *C. gattii* from 119 cloacal samples of Passerine and Psittacine without success (data not shown), as the results of recent reports that obtained null or few isolates from cloacal samples [26, 27]. This low rate of isolation may be due to improper conditions of growth including the high internal temperature of birds, the high concentration of ammonia [26, 27], or glucose lacks [13] in fresh droppings. Birds generally do not manifest a true infection probably because of their relatively high body temperature and resistance to infection [3].

All isolates showed melanin production in bird-seed agar, urease production, sensitivity to cycloheximide, and growth at 37°C; they assimilated inositol, dulcitol, dextrose, maltose, sucrose, xylose, trehalose, and peptone and did not assimilated lactose, melibiose, and potassium nitrate. The isolates varied in their abilities to assimilate cellobiose, galactose, and ramnose (data not shown). All isolates from excreta were *C. neoformans* according to biotyping in CGB agar and multiplex PCR (Fig. 1). The multiplex PCR primers used were able to differentiate the two species *C. neoformans* and *C. gattii* and the results agreed with CGB results, according to Casali et al. [28].

The microsatellite-specific primer M13 and the minisatellite-specific primer $(GACA)_4$ were used to amplify DNA polymorphisms within the genome of the 36 isolates. Representative gels can be seen in Fig. 2. PCR-fingerprinting with these primers grouped all environmental isolates as molecular type VNI (serotype A, var. *grubii*). The high pH of bird's excreta [11] or presence of carbon sources [13] can explain the presence of *C. neoformans* var. *grubii*. All the isolates belonged to mating type α as determined by the amplification of a 101-bp MATα amplicon by PCR [23] (data not shown).

Out of the 25 clinical samples, 17 (68%) were obtained from HIV-positive patients, confirming that HIV is the most important risk factor for developing cryptococcosis. Of the clinical isolates, only one was recovered from a patient with no reported risk factors. The latter isolate belonged to *C. gattii*. Table 2 lists the clinical isolates, the data from patients, and results of the molecular analyses. The majority of the isolates were from cerebral spinal fluid and blood, because the disease frequently manifests as meningitis [29]. Cryptoccocal clinical isolates were more frequently obtained from males. The male-to-female ratio was 2.12:1. The age of the patients infected with cryptococcosis ranged from 17 to 57 years old (range: 34.48 ± 10.26). Almost all clinical samples, except one, were recognized as *C. neoformans* by CGB medium and multiplex PCR. PCR fingerprinting separated clinical isolates into two molecular types, all of which belonged to VNI (serotype A, var. *grubii*), except one that was a *C. gattii*, VGI type. All clinical isolates belonged to mating type α (Table 2).

Our results are in agreement with previous studies that have established the molecular type VNI as the most common type among *Cryptococcus* isolates

Table 2 *Cryptococcus neoformans* and *C. gattii* isolates from clinical samples from Mycology Laboratory, Hospital de Clinicas (UFPR), Paraná state, Brazil

Sample code	Sex	Age (years)	Risk factor	Material	Identification	Molecular/mating type
307	F	31	–	CSF	*Cn* var. *grubii*	VNI/α
308	F	22	HIV	Blood	*Cn* var. *grubii*	VNI/α
309	F	36	HIV	CSF	*Cn* var. *grubii*	VNI/α
310	M	30	HIV	CSF/blood	*Cn* var. *grubii*	VNI/α
311	M	34	HIV	CSF	*Cn* var. *grubii*	VNI/α
312	M	39	HIV	CSF	*Cn* var. *grubii*	VNI/α
313	M	38	HIV	CSF	*Cn* var. *grubii*	VNI/α
315	F	38	HIV	CSF	*Cn* var. *grubii*	VNI/α
316	M	30	HIV	CSF	*Cn* var. *grubii*	VNI/α
317	M	42	HIV	CSF	*Cn* var. *grubii*	VNI/α
318	M	32	HIV	CSF	*Cn* var. *grubii*	VNI/α
319	M	29	HIV	CSF	*Cn* var. *grubii*	VNI/α
320	M	–	–	CSF	*Cn* var. *grubii*	VNI/α
322	F	17	IMU	Blood	*Cn* var. *grubii*	VNI/α
323	M	33	HIV	CSF	*Cn* var. *grubii*	VNI/α
324	M	36	HIV	CSF	*Cn* var. *grubii*	VNI/α
325	F	23	HIV	CSF	*Cn* var. *grubii*	VNI/α
326	M	19	Hodgkin's	CSF	*Cn* var. *grubii*	VNI/α
329	M	28	HIV	Blood	*Cn* var. *grubii*	VNI/α
330	M	52	–	CSF	*C. gattii*	VGIII/α
331	M	41	–	CSF	*Cn* var. *grubii*	VNI/α
332	M	–	–	CSF	*Cn* var. *grubii*	VNI/α
333	F	57	IMU	CSF	*Cn* var. *grubii*	VNI/α
334	F	54	HIV	CSF	*Cn* var. *grubii*	VNI/α
335	M	32	HIV	CSF	*Cn* var. *grubii*	VNI/α

F, Female; M, Male; IMU, Therapy with immunossuppressive drugs; CSF, Cerebrospinal fluid; *Cn*, *Cryptococcus neoformans*

[9, 29]. A number of epidemiological studies have demonstrated that almost all cryptococcal infections in AIDS patients are due to var. *grubii*, even in the *C. gattii*-endemic areas [9, 29]. All environmental and clinical isolates were mating type α which is in accordance with the reports that this mating type is more prevalent than those of the MATa [16, 29].

Several studies have attempted to determine the relationship between environmental and clinical strains. Molecular techniques have demonstrated that isolates obtained from bird excreta and from patients are closely related [5, 18, 19]. This study and its results provide the first general overview of the molecular type of *C. neoformans* in Paraná state, in the southern region of Brazil. This preliminary data could serve as a basis for an investigation of a larger number of clinical and environmental isolates in the future.

This is the first isolation of *C. neoformans* from Passerine and Psittacine excreta in the Brazilian state of Paraná. Our findings emphasize the importance of Passerine and Psittacine bird species in the contamination of the public and domestic environments and reinforce that bird excreta is a dangerous reservoir and potential source of inhaled *C. neoformans*. The environmental isolates consisted of the same variety of the clinical isolates, suggesting that environmental sources can contribute to the development of cryptococcosis among immunocompromised patients. Considering the incidence of human cryptococcosis in Brazil and the fact that pet birds are common in this country, our results emphasize the need for

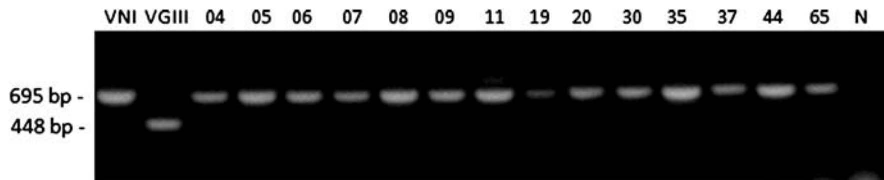

Fig. 1 *Cryptococcus* species identification by multiplex PCR. Agarose gel electrophoresis of products amplified by the multiplex PCR using the primers CNA70A and CNA70S (specific for *C. neoformans*) and CNB49A and CNB49S (specific for *C. gattii*). The predicted molecular size is indicated on the left. Left to right: *C. neoformans* WM148, *C. gattii* WM161, excreta isolates (samples number are according to Table 1), and negative control (N)

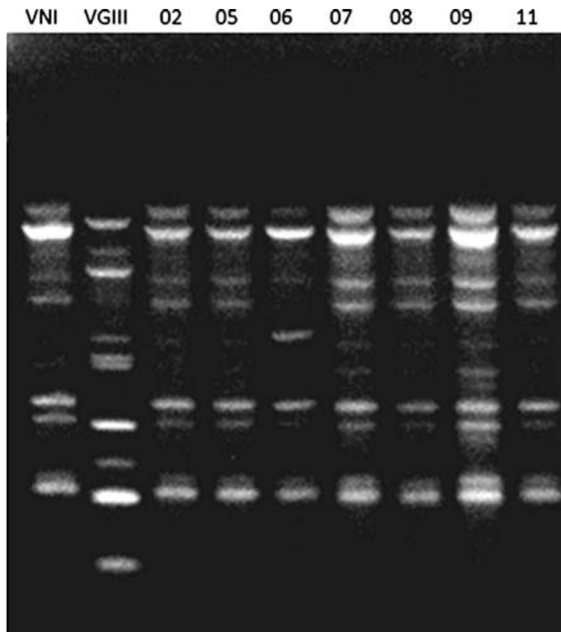

Fig. 2 Representative gel of PCR fingerprinting patterns from environmental *C. neoformans* isolates with minisatellite M13. Left to right: reference strains *C. neoformans* WM148 VNI, *C. gattii* WM161 VGIII, excreta isolates (samples number are according to Table 1), and negative control (N)

further studies to better understand the epidemiology of this fungus.

The present investigation further contributes to the characterization of isolates of *C. neoformans* in the southern region of Brazil and to the understanding of the possible epidemiological relationships between clinical and environmental isolates. It also adds information to the ecology and epidemiology of cryptococcosis. Our findings demonstrate that *C. neoformans* var. *grubii* is prevalent in clinical and environmental isolates of Paraná State and that its occurrence does not differ remarkably from what has been reported in the majority of Brazil's regions.

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