



Note

Long-term survival of *Cryptococcus neoformans* and *Cryptococcus gattii* in stored environmental samples from Colombia



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ABSTRACT

Background: Both *Cryptococcus neoformans* and *Cryptococcus gattii* have been isolated from a variety of environmental sources in Colombia.

Aim: To determine the viability of *C. neoformans*/*C. gattii* isolates in stored soil samples, filtrates and bird droppings from which these yeasts were previously recovered.

Methods: A total of 964 samples collected between 2003 and 2009, and kept at room temperature were processed. From them, 653 samples were from trees decaying wood, 274 from soil filtrates and 37 from bird droppings. When *C. neoformans* or *C. gattii* were recovered, the molecular type of each isolate was established by PCR fingerprinting using the single primer (GTG)₅.

Results: Among the processed samples, 161 isolates were recovered. From those, 81 (50.3%) corresponded to *C. gattii* recovered from decaying wood of *Eucalyptus* spp., *Corymbia ficifolia*, *Terminalia catappa* and *Ficus* spp. trees, and 80 (49.7%) corresponded to *C. neoformans* recovered from *Ficus* spp. and eucalyptus trees, as well as from bird droppings. The most prevalent molecular type among the *C. gattii* and *C. neoformans* isolates was VGII and VNI, respectively.

Conclusions: The re-isolation of *C. neoformans*/*C. gattii* from 10-year stored samples suggests that these yeasts are able to keep viable in naturally colonized samples.

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Supervivencia a largo plazo de las especies *Cryptococcus neoformans* y *Cryptococcus gattii* en muestras ambientales almacenadas procedentes de Colombia

RESUMEN

Antecedentes: *Cryptococcus neoformans* y *Cryptococcus gattii* han sido aislados de diversas fuentes ambientales en Colombia.

Objetivos: Determinar la viabilidad de *C. neoformans*/*C. gattii* en muestras de suelo, filtrados y excrementos de aves en las que se habían aislado las levaduras previamente.

Métodos: Se procesaron 964 muestras recogidas entre los años 2003 y 2009, y almacenadas a temperatura ambiente. De estas, 653 muestras provenían de detritos, 274 de filtrados de suelo y 37 de excrementos de aves. Una vez recuperados *C. neoformans* y *C. gattii*, se determinó el patrón molecular mediante la técnica de PCR huella digital con el iniciador (GTG)₅.

Resultados: Entre las muestras procesadas, se recuperaron 161 aislamientos. De estos, 81 (50,3%) fueron *C. gattii* recuperados de detritos de *Eucalyptus* spp., *Corymbia ficifolia*, *Terminalia catappa* y *Ficus* spp., y 80 (49,7%) eran *C. neoformans* recuperados de *Ficus* spp. y eucaliptos, así como de excrementos de aves. El patrón molecular más prevalente entre *C. gattii* y *C. neoformans* fue VGII y VNI, respectivamente.

Conclusiones: El reaislamiento de *C. neoformans*/*C. gattii* de muestras almacenadas durante 10 años evidencia que estas levaduras son capaces de mantenerse viables en muestras colonizadas naturalmente.

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Several studies carried out in Colombia have provided valuable data about the ecology of *Cryptococcus neoformans* and *Cryptococcus gattii*. The first environmental isolation of this pathogen was reported when *C. neoformans* serotype A was recovered from environmental samples in Bogota, Colombia, in the 1980s.³ Later, when Ellis and Pfeiffer demonstrated an association between *C. gattii* and *Eucalyptus* spp.,⁴ we conducted an environmental search in the city of Cúcuta, where an important number of clinical cases caused by this species had been reported in immunocompetent patients.¹⁰ At that time, more than 1500 samples were collected over a period of 11 months, and for the first time in Colombia and worldwide, *C. gattii* serotype C was recovered from environmental samples.¹ Recently, we reported serotype B isolates recovered from soil samples associated with *Ficus* spp. in the same city.¹² Additionally, serotype B was recovered from eucalyptus samples (*Eucalyptus* spp.) in a study undertaken in a forest area in the department of Cundinamarca.^{7,21} Furthermore, in a follow up study undertaken in 26 country areas, we analyzed data regarding samples recovered from pigeon droppings, eucalyptus and almond trees, and we found that nearly half of these sampling areas were positive for these two species of yeasts.¹⁴ More recently, *C. gattii* molecular type VGIII was recovered from *Eucalyptus ficifolia* (*Corymbia ficifolia*) detritus, which made it the first worldwide report on this association.⁹

In this study we aimed to determine the viability of *C. neoformans* and *C. gattii* isolates in naturally colonized samples associated to soil, trees decaying wood and bird droppings stored since 2003.

Materials and methods

A total of 964 samples were collected between 2003 and 2009 (Table 1) and stored at room temperature in three forms: trees decaying wood ($n = 653$), filtrates from soil ($n = 274$), and bird droppings ($n = 37$). Using conventional techniques,²⁴ 5 g of decaying wood or bird droppings were suspended in 25 ml of phosphate buffer saline (PBS), and then mixed and filtered with sterile gauze. This filtrate was mixed with 100 μ l of chloramphenicol (40 g/l), and 100 μ l of this suspension were plated onto modified *Guizotia abyssinica* agar plates with biphenyl, chloramphenicol and amikacin sulfate. The same procedure was applied to the stored filtrates. Plates were incubated at 27 °C and observed daily during up to one month for growth and colony development. A maximum of 30 colonies suspected to be *C. neoformans* and/or *C. gattii* were streaked from each plate onto Sabouraud glucose agar plates and incubated for 48 h at 27 °C. Isolated colonies were tested using conventional techniques such as urease production, assimilation of nitrogen sources and growth on canavanine glycine bromothymol blue agar medium.^{15,16} Recovered isolates were kept on glycerol (10%) at –70 °C.

Yeast concentration in each positive sample was estimated from the number of colonies observed in the *Guizotia* plates and expressed as colony forming units (CFU) per gram of sample.

For the molecular type determination, high molecular weight DNA was extracted as previously described.¹¹ DNA concentration was determined through spectrophotometry at 260/280 nm.

The following reference strains of the eight *C. neoformans* and *C. gattii* major molecular types were used: 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 175 (serotype B, VGIII) and WM 779 (serotype C, VGIV).^{8,18}

The molecular type of each isolate was determined by PCR fingerprinting using the microsatellite specific sequence (GTG)₅ as single primer, according to a slightly modified method previously described.^{8,18} Amplification products were visualized in 1.4% agarose gels in 1X TBE buffer stained with 0.3 mg/ml ethidium

bromide at 80 V for 3 h. The bands were visualized under UV light. A 1 kb molecular size marker (Promega) was loaded and the molecular types (VNI–VNIV and VGI–VGIV) were assigned by comparison to the reference strains of the eight major molecular types loaded in each gel.

Results

An overall positivity of 3.9% was obtained from the 964 samples processed; positive samples had been stored as decaying wood (52.6%), soil filtrates (36.9%), or bird droppings (10.5%). From them, 161 isolates were recovered and identified as *C. neoformans* (49.7%) and *C. gattii* (50.3%), based on the ability of *C. gattii* to assimilate canavanine in CGB medium, unlike *C. neoformans*.^{15,16} *C. gattii* isolates were recovered from samples of *Eucalyptus* spp., *C. ficifolia*, *Terminalia catappa* (tropical almond tree) and *Ficus* spp. decaying wood. Sixteen (19.7%) isolates, recovered mostly from *Ficus* spp., were typed as VGI; 43 (53.1%) isolates recovered from eucalyptus trees, as VGII, and 22 (27.2%) recovered from *C. ficifolia*, tropical almond trees and *Ficus* spp., as VGIII isolates.

C. neoformans isolates were recovered from *Ficus* spp., *Eucalyptus* spp. and bird droppings. Molecular type VNI was observed in 78 (97.5%) isolates recovered from all the above-mentioned sources; the remaining 2 isolates recovered from *Ficus* spp. were VNII. In 2 samples recovered from *Ficus* spp. both *C. neoformans* and *C. gattii* were found. CFU were estimated between 0.5×10^2 and 11.4×10^4 CFU/g of sample. Table 1 summarizes the results obtained.

Discussion

As *C. neoformans* and *C. gattii* have been successfully isolated from avian habitats and from a significant variety of tree species,^{1,3,5–7,9,12,13,17,21,23} and given that during their saprobe growth stage blastoconidia may become airborne particles that are inhaled by humans or animals, which may result in pulmonary infection, it is very important to gain insight regarding their viability in the environment.

In this brief observation, it is clear that both *C. neoformans* and *C. gattii* are able to survive in environmental samples, that had been stored at room temperature, either as soil/plant material or as filtrates from the samples. Several studies have demonstrated the ability of cells to produce melanin under natural conditions, which protects them from adverse environmental conditions such as degradative enzymes, high temperatures and ultraviolet light, as well as from phagocytic environmental predators such as amoebae.^{19,20,22} As stated before 1999, *C. gattii* seemed to be restricted to eucalyptus trees in the tropics and subtropics, while *C. neoformans* was mostly associated to pigeon droppings with a rather worldwide distribution.^{5,6,19} However, when re-processing samples previously collected, it is possible to establish that both species can be isolated from the same sample and tree species; this shows the importance of isolating and characterizing as many colonies as possible from those suspected to be *C. neoformans* or *C. gattii* in the same sample.

Specific environmental conditions are necessary for these species to exist in a particular environment, obtain nutrients, elude predators and reproduce; therefore, their survival and abundance in a niche depend on a series of factors that are essential for their viability. In this study we found that *C. neoformans* and *C. gattii* can survive in stored samples for as long as 9–10 years. Further studies are needed to determine how virulence factors express in these isolates and compare their expression levels with those of the strains initially recovered.

Table 1
Environmental recovery of isolates of the *C. neoformans*/*C. gattii* species complex in stored samples.

Sampling year (reference)	Source	Species recovered	Previous study n (% positive)	This study n (% positive)			Total isolates recovered (this study)	CFU/g of sample (this study)
				Trees decaying wood	Soil filtrates	Bird droppings		
2003 (21)	<i>Eucalyptus</i> spp.	<i>C. gattii</i>	195 (23.6)	146 (3.4)	146 (4.1)	ND	50	4.5×10^2 – 4.8×10^3
2004–2006 (7)	Almond trees	<i>C. gattii</i>	40 (ND)	40 (2.5)	ND	ND	12	11.4×10^4
2005, 2007 (9,13)	<i>Ficus</i> spp.	<i>C. neoformans</i>	128 (11.7)	ND	37 (5.4)	ND	2	1.5×10^2
		<i>C. gattii</i>						
	<i>Corymbia ficifolia</i>	<i>C. gattii</i>			91 (6.6)	ND	6	0.5×10^2 – 1×10^2
2008–2009 (12)	<i>Ficus</i> spp.	<i>C. neoformans</i>	467 (0.8)	467 (3)	ND	ND	70	0.5×10^2 – 1.1×10^3
		<i>C. gattii</i>						
	Bird droppings	<i>C. neoformans</i>	37 (0)	ND	ND	37 (10.8)	21	
Total			867	653	274	37	161	

ND: no data available.

Although conventional techniques for the recovery of this fungi from environmental samples are adequate, the standardization of a molecular test will be very useful for their detection in samples, especially in those with very low yeast concentration. A first attempt was made by our group in 2004, in which a technique for the molecular detection of the yeast in environmental samples was standardized; *Cryptococcus* spp. DNA was extracted from environmental samples and amplified with a specific PCR reaction.² Our purpose, therefore, is to standardize a PCR using these naturally colonized samples for the detection of *C. neoformans* and *C. gattii* directly from the environment.

Conflict of interests

The authors declare that no competing interests existed.

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