

COMUNICACIÓN BREVE

First environmental isolation of *Cryptococcus gattii* serotype B, from Cúcuta, Colombia

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Introduction. In Cúcuta, *Cryptococcus gattii* serotype B is commonly recovered from immunocompetent patients with cryptococcosis, but it has not been recovered from the environment in spite of its high incidence which is 77% out of reported cases.

Objective. The aim of this work was to carry out an extensive environmental sampling in Cúcuta, in an attempt to isolate *C. gattii* serotype B and to expand our knowledge about the ecology and epidemiology of this important yeast.

Materials and methods. Samples associated with 3,634 trees from 40 zones of Cúcuta were collected and processed with 28 samples collected near the houses of four patients with cryptococcosis caused by *C. gattii* serotype B. The serotype of the recovered isolates was done using multiplex PCR, molecular patterns were determined by RFLP of the *URA5* gene and mating type was determined using the primers MfaU, MfaL, Mfa2U and Mfa2L.

Results. In total, 4,389 samples were processed and one isolate of *C. gattii* serotype B (VG1/a), two isolates of *C. gattii* serotype C (VGIII/a) and three isolates of *C. neoformans* var. *grubii*, serotype A (VNI/a), were recovered. The density of the recovered isolates varied from 50 to 350 cfu/g of soil.

Conclusions. This is the first report on the environmental isolation of *C. gattii* serotype B from Cúcuta. However, because of the low rate of recovery of isolates from soil only, the environmental niche of *C. gattii* has not been established and further environmental studies in Cúcuta are necessary, owing that this serotype is not only causing cryptococcosis but also has shown a higher virulence after the Vancouver outbreak.

Key words: *Cryptococcus*, mycoses, environment, ecology, Colombia, sampling studies.

Primer aislamiento ambiental de *Cryptococcus gattii* de serotipo B, en Cúcuta, Colombia

Introducción. En Cúcuta, *Cryptococcus gattii* de serotipo B se recupera comúnmente de pacientes inmunocompetentes con criptococosis, pero no ha sido recuperado del ambiente a pesar su alta incidencia, que es de 77 % de los casos reportados.

Objetivo. El objetivo de este trabajo fue realizar un extenso muestreo ambiental en Cúcuta, para aislar *C. gattii* de serotipo B y expandir nuestro conocimiento sobre la ecología y la epidemiología de esta importante levadura.

Materiales y métodos. Se recolectaron y se procesaron muestras asociadas con 3.634 árboles de 40 zonas de Cúcuta y 28 muestras cercanas a las casas de cuatro pacientes con criptococosis causada por *C. gattii* de serotipo B. El serotipo de los aislamientos recuperados se estableció usando PCR múltiple, el patrón molecular se determinó por RFLP del gen *URA5* y la pareja sexual se determinó usando los iniciadores MfaU, MfaL, Mfa2U y Mfa2L.

Resultados. Se procesaron 4.389 muestras y se recuperó un aislamiento de *C. gattii* de serotipo B (VG1/a), dos aislamientos de *C. gattii* de serotipo C (VGIII/a) y tres aislamientos de *C. neoformans* var. *grubii* de serotipo A (VNI/a). La densidad de los aislamientos recuperados varió entre 50 y 350 UFC/g de suelo.

Conclusiones. Éste es el primer reporte de la recuperación ambiental de *C. gattii* de serotipo B en Cúcuta. Sin embargo, por la baja tasa de recuperación de aislamientos únicamente en suelo, el nicho ambiental de *C. gattii* no se pudo establecer, por lo que es necesario realizar posteriores estudios ambientales en Cúcuta, debido a que este serotipo, no sólo está causando criptococosis, sino también muestra una gran virulencia después del brote ocurrido en Vancouver.

Palabras clave: *Cryptococcus*, micosis, ambiente, ecología, Colombia, muestreo.

Cryptococcus neoformans/*Cryptococcus gattii* species complex comprises a group of capsulated yeasts causing cryptococcosis in humans and animals worldwide (1). *C. neoformans* var. *grubii*, serotype A and *C. neoformans* var. *neoformans*, serotype D, have been mostly isolated from immunocompromised host with disseminated cryptococcosis, while *C. gattii*, serotypes B and C is most often associated with neurological disorders in hosts with normal immunity (2-4) and with disease acquired via inhalation of basidiospores from the environment, which are the principal infectious propagules for the *C. neoformans*/*C. gattii* species complex (8).

In Colombia, *C. neoformans* var. *grubii* is the most important cause of cryptococcosis, followed by *C. gattii* serotype B (9), which is mainly recovered in patients from Cúcuta, where this serotype is the most frequently isolated in immunocompetent patients (10). However, while *C. gattii* serotype C, has been isolated in Cúcuta from almond trees (*Terminalia catappa*) (11), *C. gattii* serotype B has not been recovered from the environment in spite of the high incidence of this serotype associated with cryptococcosis, which is 77% out of the reported cases in immunocompetent patients (9,10). Additionally, the vast majority of clinical isolates of *C. gattii* serotype B recovered in Cúcuta since 1990, belong to the molecular type VGII (12), the same molecular type of the high virulence isolates associated with the Vancouver Island outbreak (13), where an increasing number of cases of cryptococcosis in both human and animals caused by *C. gattii* serotype B have been reported (14).

On the other hand, *C. gattii* serotype B has been isolated several times from different environmental sources in the world (14-17); for that reason, the aim of this work was to carry out an extensive environmental sampling survey in different areas of the city of Cúcuta, in an attempt to isolate *C. gattii* serotype B from different trees, to determine its circulation and to expand our knowledge about the ecology and the epidemiology of this pathogenic yeast in our country.

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Materials and methods

Study area

Cúcuta is the capital city of the department Norte de Santander, in the north-east of Colombia, limiting with Venezuela. It is situated at 7° 54'48" north latitude and 72°30'36" west longitude, at 320 m above sea level and the average temperature is 28 °C with an annual rainfall of 763 mm approximately (18,19).

Sample collection

Samples including soil (n= 3,634), bark (n= 358), detritus (n= 225), leaves (n= 103) and fruits (n= 69) were collected and processed from 3,634 trees from Cúcuta, including, mainly, the species *T. catappa* (almond tree), *Ficus* sp., *Licania tomentosa* (oiti), *Pithecellobium dulce*, *Fagara rhoifolia*, *Melicoccus bijugatus* (mamón), *Enterolobium cyclocarpum* and *Mangifera indica* (mango). No swab samples were taken. Extensive collections were made in January, May and August 2008, from 40 zones of the city, which were defined by map grids (18) and included both urban and rural areas. Data of temperature, climatic conditions as rainfall and relative humidity, and season of the year were considered for the analysis of the results (19). In the zones where either *C. neoformans* or *C. gattii* was found, a sampling was made monthly from August 2008 to February 2009, to increase the number of isolates recovered. Additionally, 28 samples were collected from 22 trees near the houses of four patients with cryptococcosis caused by *C. gattii* serotype B molecular type VGII.

Sample processing

Five grams of each sample were suspended in 25 ml of phosphate buffer saline (PBS), mixed and filtered with sterile gauze. Fifteen ml of the filtrate were mixed with 50 µl of a solution of penicillin (20 U) and streptomycin (40 U) and 100 µl of this suspension was plated in *Guizotia abyssinica* agar, supplemented with penicillin and streptomycin (20). Plates were incubated at 27 °C for one month, with weekly observation. Brown colonies grown in the media were isolated and transferred to a new *G. abyssinica* agar and next to Sabouraud dextrose agar for their identification using conventional techniques (21). Colony forming units (cfu) were counted and their number calculated and expressed per gram of sample, considering the volume plated and the dilutions of the filtrates. Additionally, all colonies from the same sample were taken to identify *C. neoformans*/*C. gattii* isolates.

Typing

DNA of the isolates of the *C. neoformans/C. gattii* species complex recovered from the environment was extracted as previously described (22). Serotype was determined using the multiplex PCR described by Ito-Kuwa *et al.*, with some modifications (23). The primers *LAC1* No 2 and *LAC1* No 4 and the new primers *LAC1* No 5 (5'-CAGCATTGTGAGCGCTTTTCCG) and *LAC1* No 6 (5'-GCATTACTGTGAGTGTACTTATAG) were used and PCR amplification was initiated at 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 46 °C for 40 s and 72 °C for 90 s, and then 7 min of extension at 72 °C, in a thermal cycler MJ Mini (Bio-Rad Laboratories). The amplified products were separated by 2% agarose gel electrophoresis at 100 V for 1 h and the gels were stained with ethidium bromide and photographed under ultraviolet light. Strains WM 628 serotype A, WM 629 serotype D, H0058-I-455 serotype C and H0058-I-2976 serotype B were used as controls.

Molecular type and mating type determination

Restriction fragment length polymorphism (RFLP) of the *URA5* gene was used as the molecular typing technique as previously reported by Meyer, *et al.* (24). The *URA5* gene was amplified with two primers: *URA5*(5'ATGTCCTCCCAAGCCCTCGACTCCG3') and SJ01 (5'TTAAGACCTCTGAACACCGTACTC3'). The amplification products were double digested with *SAU961* (10 U/μl) and *HhaI* (20U/μl) overnight and separated by 2.5% agarose gel electrophoresis at 80 V for 3.5 h using a 50 bp molecular size marker (Promega). RFLP patterns were assigned by comparing them with the eight molecular types VNI -VNIV and VGI - VGIV (24,25). Mating type was determined using the primers MfaU, MfaL, Mfa2U and Mfa2L and PCR reactions and amplifications were performed as previously published (26).

Results

In total, 4,389 samples were processed from the 3,634 trees studied and among all samples processed, three (0.07%) were positive for *C. gattii* and three (0.07%) for *C. neoformans*.

From the 3 samples positive for *C. gattii*, seven *C. gattii* serotype C, molecular type VGIII isolates and one *C. gattii* serotype B, molecular type VGI isolate were recovered (Figure 1). From the 3 samples positive for *C. neoformans*, 13 *C. neoformans* var. *grubii*, serotype A, molecular type VNI isolates were recovered. Additionally, all *C. neoformans*

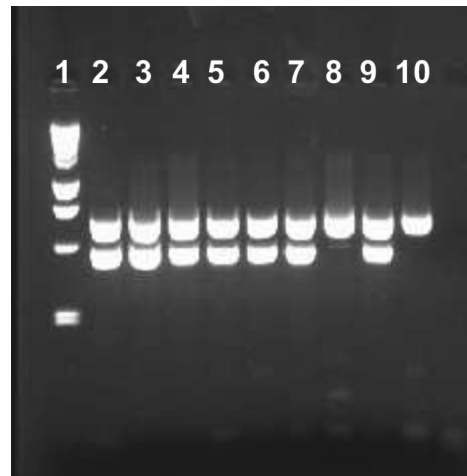


Figure 1. Typing of the isolates of the *Cryptococcus gattii* recovered from soil associated with *Ficus* sp. in Cúcuta, 2008-2009.

Lane 1: weighting marker 1 Kb, lanes 2-7: isolates H0058-I-3026, H0058-I-3027, H0058-I-3077, H0058-I-3078, H0058-I-3079, H0058-I-3081 (*Cryptococcus gattii* serotype C), lane 8: isolate H0058-I-3082 (*C. gattii* serotype B), lane 9: control strain H0058-I-455 (serotype C), and lane 10: control strain H0058-I-2976 (serotype B).

var. *grubii* and *C. gattii* serotype C isolates were mating type alpha, while the only *C. gattii* serotype B isolate was mating type a.

Among the recovered isolates, in January 2008, five colonies (250 cfu/g) of *C. neoformans* var. *grubii* and one colony (50 cfu/g) of *C. gattii* serotype B were recovered simultaneously from the same sample of soil associated with a *Ficus* sp. tree in the San Eduardo zone. In May 2008, one colony (50 cfu/g) of *C. gattii* serotype C was recovered in La Libertad zone from soil, and, in the Estadio General Santander, one colony (50 cfu/g) of *C. neoformans* var. *grubii* and six colonies (300 cfu/g) of *C. gattii* serotype C, were found simultaneously in the same sample of soil in a tree of *Ficus* sp. Due to these findings, the Estadio General Santander, San Eduardo and La Libertad zones were sampled again in August 2008. In this last sampling, only seven colonies (350 cfu/g) of *C. neoformans* var. *grubii* were recovered from soil in the Estadio General Santander (Table 1), while *C. gattii* was not found. The San Eduardo zone was sampled one more time in February 2009, because of the finding of *C. gattii* serotype B, but no isolates were recovered, and among the 28 samples collected from the houses of the four patients with cryptococcosis produced by *C. gattii* serotype B, no isolates were recovered either.

Table 1. Isolates of the *Cryptococcus neoformans*/*Cryptococcus gattii* species complex recovered from soil associated with *Ficus* sp. in Cúcuta, 2008-2009.

Date	Climatic conditions T – RH	Zone	Specie	Serotype	Molecular type	Mating type
January 2008	29.8 °C - 69%	San Eduardo	<i>C. neoformans</i>	A	VNI	α
			<i>C. gattii</i>	B	VGI	a
May 2008	28.8 °C – 72%	La Libertad	<i>C. gattii</i>	C	VGIII	α
May 2008	28.8 °C – 72%	Estadio General Santander	<i>C. neoformans</i>	A	VNI	α
			<i>C. gattii</i>	C	VGIII	α
August 2008	28.1 °C – 61%	Estadio General Santander	<i>C. neoformans</i>	A	VNI	α

T: Temperature

RH: Relative humidity

There was no association between temperature, climatic conditions and season of the year for the recovering of isolates of the *C. neoformans*/*C. gattii* species complex (Table 1). Additionally, no isolates were recovered directly from bark, detritus, leaves or fruits from the trees sampled; all isolates recovered were collected from soil.

Discussion

Although the number of isolates of the *C. neoformans*/*C. gattii* species complex recovered in this study was very small, this is the first report about the isolation of *C. gattii* serotype B from the environment in Cúcuta. This report confirms the presence of *C. gattii* in a terrestrial ecosystem, and although the isolate recovered was molecular type VGI, in contradistinction to the clinical isolates that have been recovered in Cúcuta, which are the majority molecular type VGII (12), the isolation of *C. gattii* serotype B helps us to clarify how the incidence of human cases of cryptococcosis reported in this Colombian city, could be probably due to the environmental exposure to this fungus, due to the fact that the recovering of *C. gattii* from the environment in Cúcuta had been only reported for serotype C isolates (11). Additionally, this suggests that it is necessary to do further studies in order to recover environmental isolates belonging to the same molecular pattern as the clinical isolates.

It is very important to mention that the serotype identification of the serotype B isolate recovered was done using a multiplex PCR described by Ito-Kuwa S, *et al.*, which is a technique that helps us predict the serotype within the *C. gattii* isolates, and in no manner replaces the serotyping done with the specific antisera, since it does not directly detect the capsular serotype itself. However, it is worth mentioning that this multiplex PCR has been

proven to be an excellent choice for *C. neoformans* serotypes A and D. An important fact, is that all *C. neoformans* var. *grubii* isolates recovered were molecular type VNI and most of the *C. gattii* isolates were molecular type VGIII (67%), which is in agreement with other studies made in Colombia, where these molecular types had been the most prevalent within these species (12,25).

In addition, the findings of this study increase the number of reports of the recovering of *C. gattii* from the environment, although it was not possible to associate the isolate with a specific tree species, because the isolate was recovered from soil, in contrast to previous studies of ecological reservoirs of *C. gattii*, in which associations between the yeast and different trees species had been found due to the recovering of the yeast from vegetable parts (14-17,27,28). However, our results show that the soil is the sample that is harboring the fungus, while the other tree material was negative, possibly because most of the samples were taken from soil (82.8%), but suggesting that the soil is one of the main reservoirs of colonization for this pathogen, and therefore, the fungus may be interacting with non-vertebrate hosts as nematodes and amoebae, as suggested by Casadevall *et al* (29).

Our results also reinforce that *C. gattii* is not associated with a particular species of tree, or a specific niche, because it was recovered from soil, which could be possible due to the contamination with avian dropping, which is considered an important substrate for the presence and maintenance of *C. neoformans* in nature (30) and although there are no reports for the recovering of *C. gattii* from avian dropping, this source could also be important, considering that there are some reports where species of *Cryptococcus* had been recovered from birds excreta (31) and in Cucuta

there is a wide population of pigeons (*Columba livia*) and no sampling of these birds have been done.

Another factor that influenced the isolation of *C. gattii* from soil is the fact that all samples were recovered near to the soil surface, which is in agreement with other reports, where *C. gattii* has been recovered in the first 15 cm of the humic layer of soil, where the conditions of temperature, moisture, and nutrient requirements, are enough for the colonization of the fungus (32).

Compared with other studies where *C. gattii* had been found in 27.5% and 99% of the sampled trees, and the recovery was between 50 and 15 x 10⁶ cfu/g of sample (16,33) and *C. neoformans* had been recovered among 7.9% and 14% of the samples, with a recovery between 50 and 1x10⁷ cfu/g of sample (33,34), in our study, only 0.07% of the samples processed were positive for *C. neoformans/C. gattii* species complex. However, the recovery of only one isolates of *C. gattii* serotype B from all of the sampled areas, including the houses of patients with cryptococcosis, and given its prevalence in Cúcuta, could suggest that the fungus exists in few amounts in the environment in comparison with another species of bacteria or fungi which makes it difficult to be recovered, and further sampling over an extended period of time is required to find new ecological niches of *C. gattii* in this city. Besides, it is necessary to recover a higher number of isolates to associate the distribution of the fungus with climatic conditions, season of the year and temperature of the city, because in this study no association was found, due to the small number of isolates.

On the other hand, the isolation of *C. gattii* serotypes B and C with *C. neoformans* var. *grubii* from the same place, like the co-distribution of different genotypes or serotypes found in previous studies (27,28,32) could suggest a related introduction and dispersal mechanisms or synergistic colonization considering that in some cases the populations could be very complex.

In conclusion, and in spite of the low recovery rate of *C. gattii* serotype B, this study shows the importance of continuing doing environmental samplings in Cúcuta where *C. gattii* is causing cryptococcosis, due to the fact that this pathogen has shown a higher virulence after the outbreak in Vancouver Island (13) and ecological studies are relevant in the epidemiology of a pathogenic agent. In addition, the identification of new habitats of *C. gattii* is very important and it is possible that the presence of

yeasts in soil is the source of contamination of immunocompetent individuals, because the yeast can be dispersed by the air and patients acquire the cryptococcal infection from environmental sources (8,15). For further studies, other environmental sources such as pigeon droppings or biodegraded wood could be included to increase the possibility of recovering more isolates.

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Conflicts of interests

The authors declare that there are no conflicts of interest.

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