Research article

Recovery of environmental *Cryptococcus neoformans* and *Cryptococcus gattii* in Cúcuta and association with clinical isolates

Asbleide Angarita-Sánchez[1]* ✉ⒾEsp; Denny Cárdenas-Sierra[1] ✉ⒾM.Sc; Claudia Parra-Giraldo[2] ✉ⒾPh.D; Claudia Diaz-Carvajal[1] ✉ⒾM.Sc; Patricia Escandon-Hernandez[3] ✉ⒾM.Sc.

[1] Universidad de Santander, Facultad de Ciencias de la Salud, Grupo BIOGEN, Cúcuta, Colombia.
[2] Pontificia Universidad Javeriana, Facultad de Ciencias, Departamento de Microbiología, Unidad de Proteómica y Micosis Humanas, Grupo Enfermedades Infecciosas, Bogotá, Colombia.
[3] Instituto Nacional de Salud, Grupo de Microbiología, Bogotá, Colombia.
* Correspondencia: as.angarita@udes.edu.co.

ABSTRACT

Objective. Isolate, identify and molecularly characterize pathogenic *Cryptococcus* isolates for humans from environmental and clinical samples from the city of Cúcuta. **Materials and methods.** A total of 1300 samples were collected from 446 trees of 10 different species in 10 public areas of Cúcuta. Concomitantly, clinical isolates of *Cryptococcus neoformans* were obtained (June 2016-June 2017). The samples were cultivated in *Guizottia abysinica* seed medium and were then biochemically identified and characterized by PCR fingerpinting and RFLP of the URA5 gene. **Results.** *C. neoformans* displayed an environmental prevalence of 4.3% (19 positive individuals), and that of *C. gattii* was 0.2% (1 positive individual); this yielded a total of 20 *Cryptococcus*-positive trees and 21 isolates (two from the same individual). Santander Park registered 47.6% of the global prevalence (10/21 isolates), followed by La Victoria Park with 23.8% (5/21 isolates), corresponding to *C. neoformans*. One *C. gattii* isolate was collected from a *Ficus benjamina* tree located in Mercedes Ábrego Park. Genotypic analysis revealed the presence of *C. neoformans* var. *grubii* VNI in 85.7% of environmental isolates as well as 100% of clinical isolates. VNII and VGII molecular types represented 9.5% and 4.8% of the environmental isolates, respectively. **Conclusions.** The longitudinal sampling of previously reported environmental niches of the fungus reveals its presence and suggests that permanent monitoring is required both in the environment and in patients, especially in endemic areas of the city.

Keywords: Colombia, Cryptococcosis, Genotype, Habitat, Prevalence (*Source: DeCS & MeSH*).

RESUMEN

Objetivo. Aislar, identificar y caracterizar molecularmente aislamientos de *Cryptococcus* patógenos para humanos a partir de muestras ambientales y clínicas de la ciudad de Cúcuta. **Materiales y métodos.** Se recolectaron 1300 muestras de 446 árboles de 10 especies diferentes, en 10 zonas públicas de Cúcuta. Concomitantemente, se obtuvieron aislados clínicos de *Cryptococcus neoformans* (junio de 2016-junio de 2017). Se realizó cultivo en agar semillas de *Guizottia abysinica*, posterior identificación bioquímica y caracterización genética mediante PCR-huella Digital y RFLP-URA5. **Resultados.** Se determinó prevalencia ambiental para *C. neoformans* de 4.3% (19 individuos positivos) y *C. gattii* de 0.2% (1 individuo positivo), para un total de 21 aislados y 20 árboles positivos. El parque Santander registró el 47.6% de la prevalencia global (10/21 aislados), seguido del parque La Victoria con 23.8% (5/21 aislados), correspondientes a *C. neoformans*. Se obtuvo un aislado de *C. gattii* en un individuo *Ficus benjamina* del parque Mercedes Ábrego. El análisis genotípico reveló presencia de *C. neoformans* var. *grubii* VNI en el 85.7% de los aislados ambientales, así como en el 100% de los clínicos, seguido de VNII y VGII en 9.5% y 4.8% de los aislados ambientales, respectivamente. **Conclusiones.** El muestreo longitudinal de los nichos ambientales previamente reportados del hongo revela su presencia y sugiere que se requiere una vigilancia permanente tanto en el medio ambiente como en los pacientes, especialmente en las zonas endémicas de la ciudad.

Palabras clave: Colombia, criptococosis, genotipo, hábitat, prevalencia, (*Fuentes: DeCS, MeSH*).

INTRODUCTION

Cryptococcosis is a mycosis that affects immunocompetent and immunodeficient individuals, especially those with AIDS ([1],[2],[3],[4],[5],[6]), its causative agent being the encapsulated yeast *Cryptococcus* spp., with its two pathogenic species *C. neoformans* and *C. gattii*; the first one has the varieties *grubii* (corresponding to serotype A, molecular pattern VNI and VNII) and *neoformans* (corresponding to serotype D, pattern VNIV), as well as the hybrid serotype AD molecular pattern VNIII. For its part, *Cryptococcus gattii* comprises serotypes B and C (molecular pattern VGI-VGIV) ([7]). In 2015 a taxonomic review of both complexes was published, with a new proposal of seven species: *C. neoformans*, *C. deneoformans*, *C. gattii*, *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii*, and *C. decagattii* ([8]).

The study of clinical and environmental isolates of *Cryptococcus* as a pathogen in humans is relevant in the Cúcuta region. The incidence of the disease in the general population in Colombia was 0.23 cases per 100,000 people, North Santander being the department with the highest incidence: 0.56 cases per 100,000 people. *C. neoformans* var. *grubii*, with the VNI molecular pattern, is the main etiological agent of cryptococcosis in both immunocompetent and immunosuppressed individuals; this mycosis has high morbidity and mortality and has been serving as a sentinel marker for HIV infection. ([9]) Epidemiological analyzes and the constant occurrence of clinical cases compatible with cryptococcosis show Cúcuta as a city with a high prevalence of infection, mainly that caused by *C. gattii,* molecular pattern VGII ([10]).

Taking into account the association of genetic subgroup VGII with high virulence and potential to generate outbreaks, this prevalence is worrying ([10]) Although infection with *C. gattii* is a minority in Colombia with respect to *C. neoformans* (serotypes A and D), the municipality of Cúcuta has a high prevalence (60%) in HIV negative patients ([11]) and globally, a lower frequency of cryptococcosis due to *C. gattii* is reported in immunocompromised patients including HIV / AIDS, which have been associated with genotypes VGIII and VGIV, as is the case of California, USA, considered an endemic area for VGIII, with infection attributed to it in 12% of these ([12]).

From the environment, serotype C of *C. gattii* was isolated for the first time in Cúcuta in 1998 ([13]). Subsequently, in 2011 the first environmental isolation of *C. gattii* serotype B was obtained in this same city ([9]).

Regarding to the high incidence of cryptococcosis in the city of Cúcuta, the objective of the present study was to determine the presence of *C. neoformans* and *C. gattii* in the urban area of the city, and to establish the association of clinical isolates with those recovered from environmental samples.

MATERIALS AND METHODS

Study site and sample collection. We collected 1300 samples from 446 trees distributed as follows: soil (442), bark (434), nuts (40) and leaves (384); at the time of collection of the samples, the presence of feces of pigeons on their surface was selected as the main characteristic. The first sampling was carried out in October 2016 in the Mercedes Abrego, Simon Bolivar, Arcorirs (La Libertad) parks and peripheral zone to the General Santander Stadium. The second sampling was carried out in the month of January 2017 in the Parks Antonia Santos, La Victoria, Juana Rangel de Cuellar and Santander Park and the third, in the month of April 2017, was held in the National Parks and Fuente de Leones.

On the other hand, 6 isolates of *C. neoformans* cerebrospinal fluid (CSF) of HIV-positive patients with meningeal cryptococcosis were obtained from the Erasmo Meoz University Hospital of Cúcuta between June 2016 and June 2017. These samples corresponded to 4.3% of the total of those reported through the passive surveillance of cryptococcosis carried out in the country through the Epidemiological Survey on Cryptococcosis in Colombia.

Processing of environmental samples. A mapping of the sampling sites where each individual (tree) was identified was done to collect samples in each of the selected areas of the metropolitan area of Cúcuta, using plastic bags.

These were processed using a phosphate buffered saline (PBS) extraction technique and supplemented with antibiotics as previously described ([14], [15]), for which 5 g of sample was weighed, resuspended in 25 ml of 1X PBS for homogenization and allowed to rest for 60 minutes; each homogenate was subsequently filtered with sterile gauze, and an antibiotic was finally added (allowing it to act for 60 minutes).

A total of 100 µl of each preparation was planted on agar (Guizottia abysinica) and then incubated at a temperature of 27°C for 20 days, with weekly periodic observations as previously described ([16]).

Creamy elevated colonies with regular edges and brown pigment (the production of melanin) were obtained and observed under a microscope using India ink (Figure 1). Every colony, corresponding to large round yeasts with the presence of a capsule, was subjected to urea degradation tests ([17]) and determination of species using CGB agar (canavanine agar glycine blue sodium bromide) ([18]).
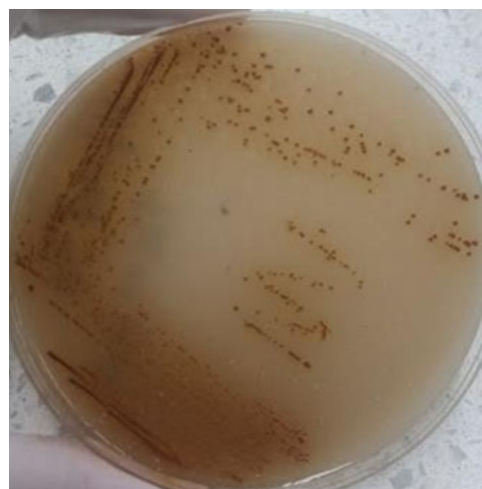

Figure 1. *Cryptococcus* sp. Agar culture on *Guizottia abysinica* agar (production of melanin). University of Santander, Cúcuta.

Processing of clinical isolates. An agar culture (Guizottia abysinica) was carried out for isolates of clinical origin (Figure 2) with urea degradation tests ([14]) and determination of species in CGB ([18]).
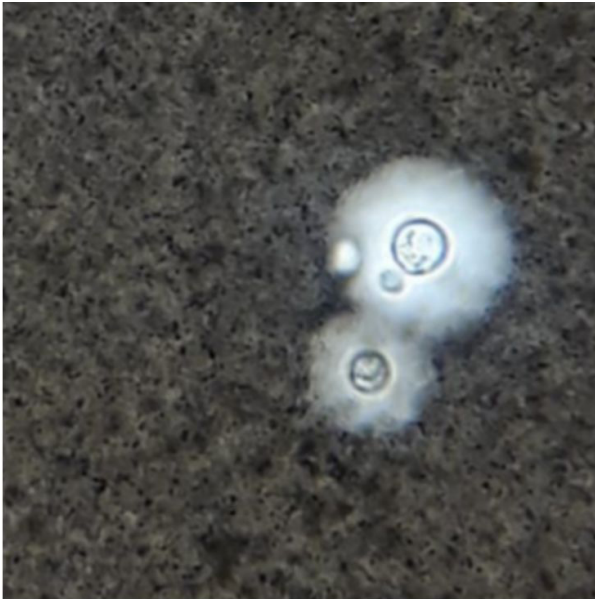

Figure 2. *Cryptococcus* sp sample of cerebrospinal fluid in India ink. Microbiology Group, National Institute of Health.

Determination of the molecular pattern by PCR–Fingerprint. The high-molecular-weight DNA was extracted using the phenol:chlororofm:isoamyl alcohol method according to Casali ([19]). This procedure was carried out using biomass obtained from the clinical and environmental strains once cultured on Saboureaud agar at 27°C for 48 hours. Subsequently, colonies were transferred from the culture to 1.5 ml microcentrifuge tubes and incubated at -20°C overnight. Then suspended in 500µl of extraction buffer (50mM Tris-HCl, 50mM EDTA, 3% sodium dodecyl sulfate, 2% mercaptoethanol), vigorously vortexed and incubated at 65°C for 1 hour. The lysate was extracted with phenol-chloroform-isoamyl alcohol (25: 24: 1, vol / vol / vol). The DNA was recovered by precipitation with isopropanol at -20°C overnight, washed with 70% ethanol (vol/vol) and then finally resuspended in TRIS-EDTA buffer (TE). The concentration was measured by fluorescence (Quibit 3.0 fluorometer).

The molecular characterization of the species of *Cryptococcus* spp, was carried out by PCR fingerprint, using as the first single specific sequence microsatellite (GTG) 5 (5'-GTGGTGGTGGTGGTG-3') described by Escandón et al in 2006 ([20]).

The 50 µL PCR mixture contained: 31.5 µL of sterile deionized water, 25 ng of DNA, 5 µL of 10X PCR buffer (10 mM Tris / HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl) (Invitrogen , Carlsbad, CA), 0.02 mM of each dNTP (dATP, dCTP, dGTP and dTTP) (Promega, Madison, WI), 3 mM sodium acetate (Sigma, Atlanta, GA), O.8 ng of primer (for GTG5 the concentration was 10 ng), 2 mM MgCl, 1 ml of bovine serum albumin (BSA, 200 mg mL^{-1}\Δ1) and 0.05 U Amplitaq. PCR was performed for 35 cycles in a SimpliAmp ™ Thermal Cycler using the following conditions: denaturation at 94°C for 20 seconds,

annealing at 50°C for 1 minute, extension at 72°C for 20 seconds and a final extension cycle at 72°C for 6 minutes. The amplification products were separated by electrophoresis in a 1.4% agarose gel in Tris-borate EDTA (TBE) 1X buffer at 100 V for 2 hours. The products of the (GTG)5 amplification were stained with Gel red Nucleid Acid Gel Stain Biotium at 0.3 mg mL^{-1}, for 30 minutes. The bands were visualized under UV light using 1 kb molecular size marker in three wells to allow the normalization of the gels. The molecular types (VNI-VNIV and VGI-VGIV) were assigned to compare the reference strains of the eight main molecular types loaded in each gel ([21]).

Determination of the molecular pattern by the RFLP-URA5 gene and association analysis. The identification of the molecular types of *Cryptococcus* spp. both the clinical and environmental strains were performed with the two primers URA 5 (5'ATGTCCTCCCAAGCCCTCGACTCCG3') and SJ01 (5'TTAAGACCTCTGAACACCGTACTC3').

The PCR reaction was carried out as described by Meyer et al ([17]); the PCR products were subjected to a double enzymatic digestion with Sau 96I and Hha I at 37°C for 3 hours. The restriction fragments were separated by electrophoresis in 3% agarose gel in Tris-borate EDTA (TBE) 1X buffer at 100 V for 5 hours. The restriction profiles were compared with the standards obtained from the reference strains, using analysis in the BioGalaxy module of the Biolomics program, version 3.0.

RESULTS

Characterization of environmental samples. A total of 75% of the trees analyzed came from 6 parks: Simón Bolívar Park with 80 individuals provided the most, followed by Santander, La Victoria, Nacional and Mercedes Abrego parks (with 70, 68, 67 and 50 individuals, respectively). This last number is the same for the area surrounding General Santander Stadium.

In turn, the diversity of the individuals analyzed in the present work comprised 10 species, with the Oití (*Licania tomentosa*) being the most widely distributed and predominant taxon with 66.4% of the total individuals, followed by the Almond tree (*Terminalia catappa*) with 13.2%, these two species comprising approximately 80% of the total sample. The remaining 20% was distributed among Mamoncillo *(Melicoccus* bijugatus) with 7.2%, Ficus (*Ficus benjamina*) with 5.4%, Tamarind (*Tamarindus indica*) with 2.9%, Chiminango (sweet Pithecellobium) and Totumo (1.8% each), Saman (*Samanea saman*), Lemon swingle (*Swinglea glutinosa*) and Mango (*Mangifera indica*) (0.4% each).

The initial culture revealed the presence of 21 isolates compatible with *Cryptococcus* spp., 20 of which corresponded to *C. neoformans* (95.2%) and 1 to *C. gattii* (4.8%), obtained from 20 of the 446 individuals, since double positivity was obtained for *C. neoformans* in the same tree (Table 1). Therefore, prevalences of 4.3% and 0.2% were obtained for *C. neoformans* and *C. gattii*, respectively.

Environmental isolates with their area of origin, species of the individual and isolation with molecular type identified by PCR fingerprint with primer (GTG) 5 and by RFLP URA5 gene are also described in Table 1.

Table 1. Description of positive samples for *C.neoformans* and *C.gattii* recovered in the city of Cúcuta, Norte de Santander.

Identification samples	Type of samples	Área of origin	Species of individual	Specie of *Cryptococcus*	Variety	Genotype *Cryptococcus*
25	bark	Santander park	Mango (*Mangifera indica*)	*C. neoformans*	*grubii*	VN I
35	bark	Santander park	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
37	soil	Santander park	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
39a*	leaves	Santander park	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
39b*	soil	Santander park	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
47	bark	Santander park	Mango (*Mangifera indica*)	*C. neoformans*	*grubii*	VN I
58	soil	Santander park	Mango (*Mangifera indica*)	*C. neoformans*	*grubii*	VN I
63	bark	Santander park	Samán (*Samanea saman*)	*C. neoformans*	*grubii*	VN I
68	bark	Santander park	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
69	soil	Santander park	Tamarindo (*Tamarindus indica*)	*C. neoformans*	*grubii*	VN I
1	bark	Victoria park	Totumo (*Crescentia cujete*)	*C. neoformans*	*grubii*	VN I
10	soil	Victoria park	Mango (*Mangifera indica*)	*C. neoformans*	*grubii*	VN II
44	bark	Victoria park	Chiminango (*Pithecellobium dulce*)	*C. neoformans*	*grubii*	VN II
45	bark	Victoria park	Limón swingle (*Swinglea glutinosa*)	*C. neoformans*	*grubii*	VN I
49	bark	Victoria park	Mamoncillo (*Melicoccus bijugatus*)	*C. neoformans*	*grubii*	VN I
2	soil	Mercedes Ábrego Park	Ficus (*Ficus benjamina*)	*C. gattii*	*gattii*	VG II
5	bark	Fuente de Leones	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
7	soil	Antonia Santos park	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
12	bark	Arcoiris park	Almendro (*Terminalia catappa*)	*C. neoformans*	*grubii*	VN I
32	soil	General Santander estadium	Oití (*Licania tomentosa*)	*C. neoformans*	*grubii*	VN I
58	bark	Simón Bolívar park	Almendro (*Terminalia catappa*)	*C. neoformans*	*grubii*	VN I

* Samples derived from the same individual

In total, Santander Park occupied the first place in prevalence with 47.6% of the 21 isolates obtained, followed by La Victoria Park (23.8%) and finally Fuente de Leones, Antonia Santos, Arcoíris (La Libertad), and Simón Bolívar parks and the surroundings of General Santander Stadium (each with 4.8% of the isolates), exclusively for *C. neoformans* var. *grubii*, since a single isolate of *C. gattii* was obtained in Mercedes Ábrego Park (4.8% of the isolates).

At the same time, the calculation of the prevalence for each public area studied revealed outstanding data, especially for two of these: Santander Park with 12.8% (9/70 individuals were positive) and La Victoria Park with 7.3% (5/68 individuals were positive), in both cases only for *C. neoformans*. Figure 3 shows the individual prevalence for the different areas evaluated.
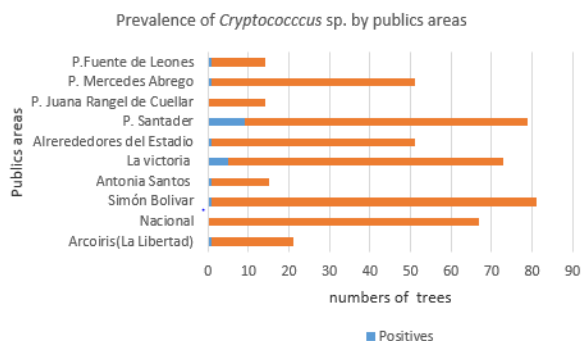

Figure 3. Prevalence of *Cryptococcus sp* in public areas areas of Cúcuta.

The positivity analysis according to the type of sample showed a predominance of *C. neoformans* isolates obtained from bark and soil with 60% (12/20 isolates) and 35% (7/20 isolates), respectively, while only one was obtained from leaves (5%). This implies a general prevalence for *C. neoformans* from bark, soil and leaves of 3.0% (of 434 samples), 1.6% (of 442 samples) and 0.3% (of 384 samples), respectively.

Regarding *C. gattii*, positivity was only obtained in a soil sample (Table 1). In general, no isolates were obtained from the 40 samples of nuts analyzed in this study.

The presence of *C. neoformans* VNI was concentrated in 55.5% in the Santander park (10/18 isolates), followed by 16.6% in Parque la Victoria (3/18 isolates) and a minority was distributed in the Fuente de Leones parks , Antonia Santos, Arcoíris (La Libertad), Simón Bolívar and surroundings of the General Santander Stadium, with 5.3% of the isolates for each zone (1/18 isolates), while the molecular type VNII was found exclusively in the Victoria Park, isolated from soil and bark of Mango tree species (*Mangifera indica*) and Chiminango (*Pithecellobium dulce*), respectively.

On the other hand, the isolate of *C. gattii* corresponded to VGII, being in turn the only one obtained from the Mercedes Abrego park, from soil associated with an individual of Ficus (*Ficus benjamina*) (Table 1).

Characterization of clinical samples. Genotypic analysis from clinical samples revealed the presence of *C. neoformans* in all cases, corresponding in their entirety to the VNI molecular type (Figure 4).
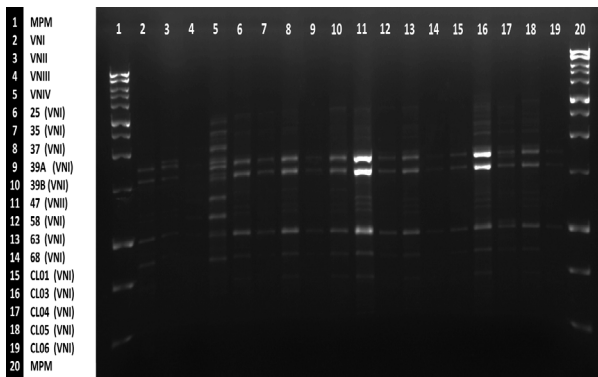
Figure 4. PCR fingerprinting using primer (GTG)5 from clinical and environmental isolates of *Cryptococcus neoformans* var. *grubii* with molecular weight marker 1kb and control strains.

Genetic relationship between *C. neoformans* isolates of clinical and environmental origin The PCR fingerprint and RFLP of the URA5 gene, revealed that the genetic pattern found in those isolates from patients is also present in most of the environmental isolates, being VNI molecular pattern the most prevalent. A correlation was observed between these two types of isolates at the genetic level/Santander park (10/18 isolates), followed by 16.6% in Parque la Victoria (3/18 isolates), and a minority were distributed among the Fuente de Leones, Antonia Santos, Arcoiris (La Libertad) , Simón Bolívar parks and surroundings of General Santander Stadium, with 5.3% of the isolates for each zone (1/18 isolates); the molecular type VNII was exclusively found in Victoria Park from the soil and bark of the Mango (*Mangifera indica*) and Chiminango (*Pithecellobium dulce*) trees, respectively.

In contrast, the isolate of *C. gattii* corresponded to VGII, being in turn the only one obtained from Mercedes Abrego Park from an individual Ficus (*Ficus benjamina*) (Table 1).

DISCUSSION

In the ecological context, there has been a predominance of *C. neoformans*, especially *C. neoformans* var *grubii* VNI and VNII (serotype A), whose habitat also extends to bird excreta ([22]). These two molecular types are also the most prevalent in cases of cryptococcosis (97%) in immunosuppressed individuals in Colombia ([21],[23]) and in other countries, including Europe ([24]). However, the environmental presence of *C. gattii* is particularly relevant to its ability to generate disease in immunocompetent individuals and its potential for adaptation to different geographical conditions, ceasing to be exclusive to tropical and subtropical areas, to also be found in temperate and rainy areas ([25]).

The present work covered 10 highly attended public areas of the city of Cúcuta, in which most of them revealed the presence of pathogens of the genus *Cryptococcus* sp, except for the National Parks and Juana Rangel de Cuéllar. The prevalence found was, in consistence with the previously mentioned ([22]), higher in general for *C. neoformans* (4.3%) with respect to *C. gattii* (0.2%) at a ratio of 21.5: 1, but on the other hand, contrasted with previous data reported for the city of Cúcuta in particular

(years 2008 and 2009), having been referred to as 0.07% in a similar way for both species of the fungus, even with a significantly greater screening of individuals (3634) from La Libertad, San Eduardo and around the General Santander Stadium ([26]).

According to our findings, the positivity for *C. neoformans* showed predominance in the Santander park (47.6% of the 21 isolates), followed by the La Victoria park (23.8%) and finally the parks Fuente de Leones, Antonia Santos, Arcoíris (La Libertad) , Simón Bolívar and surroundings of the General Santander Stadium (each with 4.8% of the isolates), exclusively for *C. neoformans grubii* variety. Taking into account the above, we can conclude that the Santander parks (especially with individual prevalence greater than 10%) and La Victoria, constitute relevant ecological niches for *C. neoformans,* the latter also providing genetic variability since both the VNI molecular type is found the VNII (only area with finding of the latter).

There was a variety of arboreal species that were found to be niches of the fungus, especially Oití (*Licania tomentosa*), widely distributed in the areas evaluated, from which 38% of the total isolates were identified (8/21 isolates), all corresponding to *C. neoformans* VNI, besides being in diversity of sources (bark, soil and leaves), followed by Mango (*Mangifera indica*), from which 4 isolates (19%) were obtained, both on soil and in bark , corresponding to *C. neoformans* variety *grubbii* (VNI and VNII) and the Almond tree (*Terminalia catappa*), with two isolates (9.5%), identified as *C. neoformans* VNI, in contrast with the previous report of the latter as habitat of both *C. neoformans* as of *C. gattii* ([25]).

In Santander Park interestingly, the five isolates obtained from the Oiti tree species (*Licania tomentosa*) corresponded to four individuals, since one of them was double positive, revealing the presence of *C. neoformans* VNI simultaneously in soil adjacent to the same and in its leaves.

This unique case of double positivity for *Cryptococcus neoformans*, recorded in the present work has no antecedent in the city, as regards the type of tree (*Licania tomentosa*).

According to the data published by Firacative et al ([26]), the tracking of more than 4300 samples from trees from different zones of Cúcuta and areas near the housing of patients infected with *C. gattii* serotype B (VGI / a), yielded only one isolate of this pathogen, two isolates of *C. gattii* serotype C (VGIII/a) and three isolates of *C. nooformans* serotype A (VNI/ a), where one of the latter, together with the only *C. gattii* isolate (VGI / a) were obtained from the same tree but in that case from Ficus species (*Ficus benjamina*) in the San Eduardo sector.

Similarly, around the General Santander Stadium, also on the soil of the same Ficus tree (*Ficus benjamina*) were *C. gattii* (VGIII / a) and *C. nooformans* serotype A (VNI/a) simultaneously isolated ([26]).

In relation to the above, the present work revealed positivity for this last species of tree, but corresponding to the isolation of *C. gattii* VGII from soil sample in the Mercedes Abrego park (without antecedent), in the heart of the downtown area of the city from Cúcuta. This was

the fourth area with the largest number of individuals screened, however, only one of its 50 trees was positive, being an individual Ficus (*Ficus benjamina*).

Ficus benjamina, harboring both *C. gattii* and *C. neoformans* (26), is important if the presence of VGI, VGII and VGIII genotypes is considered in both environmental samples and in patients with cryptococcal disease (10,21, 23). These three molecular types have also been associated with cases of human and animal cryptococcosis in the western United States. Particularly the genotypes VGII and VGIII of environmental origin have been identified as the possible etiological agent of mycosis in HIV positive individuals (27).

The findings in the area surrounding the General Santander Stadium are consistent with a previous report, which showed the presence of *C. neoformans* (26), allowing to conclude about its persistence in the area. However, this positivity was observed for the first time in trees of the species *Licania tomentosa*, reflecting a variation of both the types of habitat of the fungus, and of the species of the pathogen circulating in a given area.

Given the wide environmental distribution of *C. neoformans* and *C. gattii,* and their association to pathology in immunosuppressed and even immunocompetent individuals, the permanent tracking of common areas of high population confluence is relevant (25).

In agreement with the above, the recognition of the identity between isolates of *Cryptococcus* spp from clinical to environmental sources, by means of the comparison of circulating genotypes is of great interest taking into account also the increase of cases of cryptococcosis in HIV positive individuals, comparing the time periods 1997-2005 with the period 2006-2010 (3000 cases/million inhabitants and 3300 cases / million inhabitants, respectively) (21,23). In addition, the incidence of cryptococcosis also affects minors in Colombia (under 16 years of age), where HIV infection is a recognizable risk factor in only half of the cases, and additional factors remain to be established. It is worrisome, however, that this incidence is seven times greater for the same population group in Norte de Santander (0.122 cases/100.000 inhabitants), with respect to the national average (0.017 cases/100.000 inhabitants) (28).

The genotypic analysis of the pathogenic species from 6 clinical samples in the present work revealed exclusively the presence of *C. neoformans* var *grubii* VNI (100% of cases). Consistent with the broad prevalence registered for this in studies such as González et al (29) in Mexico, over 12 years, from 166 clinical samples (it is not specifically specified if HIV + patients), who reported prevalence for *C. neoformans* of 92.2%, with respect to *C. gattii* (7.8%) and corresponding to VNI (74.6%), VNII (9%), VNIII (4.8%), VNIV (3.6%), VGI (3.6%), VGII (1.8%), VGIII and VGIV (1.2% each) (29).

It is necessary to expand the number of clinical isolates that allow determining associations with the environmental isolates of the VNII pattern, as well as *C. gattii* present in the environment.

It is noteworthy that Cúcuta is characterized by widely forested areas and the presence of a large number of birds, mainly highlighting the C*olumbia livia* pigeon in the parks studied, being more representative the presence of these in the Santander Park where this work allows to confirm the yeast niche, other studies also show the relationship between ecological niches and these birds (30), hence the importance of a epidemiological surveillance due to the proximity to humans and the fact that they can move from a park to the other.

These data related to the high prevalence of patients with a diagnosis of cryptococcosis and its genetic relationship with environmental isolates is of great importance in public health programs.

Conflict of interests

The authors reported no potential conflict of interest

Acknowledgements

REFERENCES

1. Ye F, Xie J-x, Zeng Q-s, Chen G-q, Zhong S-q, Zhong N-s. Retrospective analysis of 76 immunocompetent patients with primary pulmonary cryptococcosis. Lung. 2012; 190(3):339-346. DOI: https://doi.org/10.1007/s00408-011-9362-8 PMid:22246551

2. Lin X, Heitman J. The biology of the Cryptococcus neoformans species complex. Annu Rev Microbiol. 2006; 60:69-105. DOI: https://doi.org/10.1146/annurev.micro.60.080805.142102 PMid:16704346

3. Perfect JR. Management of cryptococcosis: how are we doing? PLoS med. 2007; 4(2):e47. DOI: https://doi.org/10.1371/journal.pmed.0040047 PMid:17284156

4. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis. 2010; 50(3):291-322. DOI: https://doi.org/10.1086/649858 PMid:20047480

5. Alvarez-Moreno CA, Cortes JA, Denning DW. Burden of Fungal Infections in Colombia. J Fungi. 2018; 4(2):41. DOI: https://doi.org/10.3390/jof4020041 PMid:29561795 PMCid:PMC6023354

6. Esher SK, Zaragoza O, Alspaugh JA. Cryptococcal pathogenic mechanisms: a dangerous trip from the environment to the brain. Mem Inst Oswaldo Cruz. 2018; 113(7):1-15. DOI: https://doi.org/10.1590/0074-02760180057 PMid:29668825

7. Kwon-Chung KJ, Fraser JA, Doering TL, Wang ZA, Janbon G, Idnurm A, et al. Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis. Cold Spring Harb Perspect Med. 2014; 4(7):a019760. DOI: https://doi.org/10.1101/cshperspect.a019760 PMid:24985132

8. Hagen F, Khayhan K, Theelen B, Kolecka A, Polacheck I, Sionov E, et al. Recognition of seven species in the Cryptococcus gattii/Cryptococcus neoformans species complex. Fungal Genet Biol. 2015; 78:16-48. DOI: https://doi.org/10.1016/j.fgb.2015.02.009 PMid:25721988

9. Escandón P, Lizarazo J, Agudelo CI, Casta-eda E. Cryptococcosis in Colombia: Compilation and Analysis of Data from Laboratory-Based Surveillance. J Fungi. 2018; 4(1):32. DOI: https://doi.org/10.3390/jof4010032 PMid:29494502

10. Lizarazo J, Escandón P, Agudelo CI, Firacative C, Meyer W, Casta-eda E. Retrospective study of the epidemiology and clinical manifestations of Cryptococcus gattii infections in Colombia from 1997–2011. PLoS Negl Trop Dis. 2014; 8(11):e3272. DOI: https://doi.org/10.1371/journal.pntd.0003272 PMid:25411779

11. Lizarazo J, Chaves O, Pe-a Y, Escandón P, Agudelo CI, Casta-eda E. Comparación de los hallazgos clínicos y de supervivencia entre pacientes VIH positivos y VIH negativos con criptococosis meníngea en un hospital de tercer nivel. Acta Med Colomb. 2012; 37(2):49-61.

12. Chen SC-A, Meyer W, Sorrell TC. Cryptococcus gattii infections. Clin Microbiol Rev. 2014; 27(4):980-1024. DOI: https://doi.org/10.1128/CMR.00126-13 PMid:25278580

13. Callejas A, Ordonez N, Rodriguez M, Castaneda E. First isolation of Cryptococcus neoformans var. gattii, serotype C, from the environment in Colombia. Med Mycol. 1998; 36(5):341-344. DOI: https://doi.org/10.1080/02681219880000531 PMid:10075505

14. Paliwal D, Randhawa H. Evaluation of a simplified Guizotia abyssinica seed medium for differentiation of Cryptococcus neoformans. J Clin Microbiol. 1978; 7(4):346-348. PMid:357441

15. Escandón P, Quintero E, Granados D, Huérfano S, Ruiz A, Casta-eda E. Isolation of Cryptococcus gattii serotype B from detritus of Eucalyptus trees in Colombia. Biomédica. 2005; 25(3):390-397. DOI: https://doi.org/10.7705/biomedica.v25i3.1363 PMid:16276686

16. Staib F, Seibold M, Antweiler E, Fröhlich B. Staib Agar Supplemented with a Triple Antibiotic Combination for the Detection of Cryptococcus neoformtans in Clinical Specimens: Staib-Agar mit einer Dreifach-Antibiotika-Kombination für den Nachweis von Cryptococcus neoformans in klinischem Untersuchungsmaterial. Mycoses. 1989; 32(9):448-454. DOI: https://doi.org/10.1111/j.1439-0507.1989.tb02280.x PMid:2691887

17. Kwon-Chung K, Wickes BL, Booth J, Vishniac HS, Bennett JE. Urease inhibition by EDTA in the two varieties of Cryptococcus neoformans. Infect Immun. 1987; 55(8):1751-1754. PMid:3112009

18. Min K, Kwon-Chung K. The biochemical basis for the distinction between the two Cryptococcus neoformans varieties with CGB medium. Zentralbl Bakteriol Mikrobiol Hyg A. 1986; 261(4):471-480. DOI: https://doi.org/10.1016/S0176-6724(86)80079-7

19. Casali AK, Goulart L, Rosa e Silva LK, Ribeiro ÂM, Amaral AA, Alves SH, et al. Molecular typing of clinical and environmental Cryptococcus neoformans isolates in the Brazilian state Rio Grande do Sul. FEMS Yeast Res. 2003; 3(4):405-415. DOI: https://doi.org/10.1016/S1567-1356(03)00038-2

20. Escandón P, Sánchez A, Martínez M, Meyer W, Casta-eda E. Molecular epidemiology of clinical and environmental isolates of the Cryptococcus neoformans species complex reveals a high genetic diversity and the presence of the molecular type VGII mating type a in Colombia. FEMS Yeast Res. 2006; 6(4):625-635. DOI: https://doi.org/10.1111/j.1567-1364.2006.00055.x PMid:16696659

21. Escandón P, de Bedout C, Lizarazo J, Agudelo CI, Tobón Á, Bello S, et al. Cryptococcosis in Colombia: Results of the national surveillance program for the years 2006-2010. Biomédica. 2012; 32(3):386-398. DOI: https://doi.org/10.7705/biomedica.v32i3.707 PMid:23715187

22. Chowdhary A, Randhawa HS, Prakash A, Meis JF. Environmental prevalence of Cryptococcus neoformans and Cryptococcus gattii in India: an update. Crit Rev Microbiol. 2012; 38(1):1-16. DOI: https://doi.org/10.3109/1040841X.2011.606426 PMid:22133016

23. Lizarazo J, Linares M, de Bedout C, Restrepo Á, Agudelo CI, Casta-eda E. Estudio clínico y epidemiológico de la criptococosis en Colombia: resultados de nueve a-os de la encuesta nacional, 1997-2005. Biomédica. 2007; 27(1):94-109. DOI: https://doi.org/10.7705/biomedica.v27i1.236 PMid:17546227

24. Cogliati M, Zamfirova RR, Tortorano AM, Viviani MA, Network FC. Molecular epidemiology of Italian clinical Cryptococcus neoformans var. grubii isolates. Sabouraudia. 2013; 51(5):499-506. DOI: https://doi.org/10.3109/13693786.2012.751642 PMid:23286351

25. Mak S, Vélez N, Casta-eda E, Escandón P. The fungus among us: Cryptococcus neoformans and Cryptococcus gattii ecological modeling for Colombia. Journal of Fungi. 2015; 1(3):332-344. DOI: https://doi.org/10.3390/jof1030332 PMid:29376914 PMCid:PMC5753128

26. Firacative C, Torres G, Rodríguez MC, Escandón P. First environmental isolation of Cryptococcus gattii serotype B, from Cúcuta, Colombia. Biomedica. 2011; 31(1):118-123. DOI: https://doi.org/10.7705/biomedica.v31i1.342 PMid:22159490

27. Springer DJ, Billmyre RB, Filler EE, Voelz K, Pursall R, Mieczkowski PA, et al. Cryptococcus gattii VGIII isolates causing infections in HIV/AIDS patients in Southern California: identification of the local environmental source as arboreal. PLoS Pathog. 2014; 10(8):1-19. DOI: https://doi.org/10.1371/journal.ppat.1004285 PMid:25144534

28. Lizarazo J, Escandón P, Agudelo CI, Casta-eda E. Cryptococcosis in Colombian children and literature review. Mem Inst Oswaldo Cruz. 2014; 109(6):797-804. DOI: https://doi.org/10.1590/0074-0276130537 PMid:25317708

29. González GM, Casillas-Vega N, Garza-González E, Hernández-Bello R, Rivera G, Rodríguez JA, et al. Molecular typing of clinical isolates of Cryptococcus neoformans/Cryptococcus gattii species complex from Northeast Mexico. Folia Microbiol. 2016; 61(1):51-56. DOI: https://doi.org/10.1007/s12223-015-0409-8 PMid:26109075

30. Montagna MT, De Donno A, Caggiano G, Serio F, De Giglio O, Bagordo F, et al. Molecular characterization of Cryptococcus neoformans and Cryptococcus gattii from environmental sources and genetic comparison with clinical isolates in Apulia, Italy. Environ Res. 2018; 160:347-352. DOI: https://doi.org/10.1016/j.envres.2017.09.032 PMid:29054089