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CHARACTERIZATION OF *Cryptococcus neoformans* ISOLATED FROM URBAN ENVIRONMENTAL SOURCES IN GOIÂNIA, GOIÁS STATE, BRAZIL

Cláudia Castelo Branco Artiaga KOBAYASHI(1), Lúcia Kioko Hasimoto e SOUZA(1), Orionalda de Fátima Lisboa FERNANDES(1), Sula Cristina Assis de BRITO(1), Ana Cláudia SILVA(1), Efigênia Dantas de SOUSA(2) & Maria do Rosário Rodrigues SILVA(1)

SUMMARY

Cryptococcus neoformans is an opportunistic fungal pathogen that causes meningoencephalitis as the most frequent clinical presentation in immunocompromised patients, mainly in people infected by HIV. This fungus is an environmental encapsulated yeast, commonly found in soil enriched with avian droppings and plant material. A total of 290 samples of pigeon and the other avian droppings, soil, ornamental trees and vegetable material associated with *Eucalyptus* trees were collected to study environmental sources of *Cryptococcus* species in Goiânia, Goiás State. The determination of varieties, serotypes and the susceptibility *in vitro* to fluconazole, itraconazole and amphotericin B of *C. neoformans* isolates were performed. *C. neoformans* var. *grubii* (serotype A) was found in 20.3% (36/177) of pigeon dropping samples and in 14.3% (5/35) of samples of *Eucalyptus*. None of the environmental isolates of *C. neoformans* showed *in vitro* resistance to three antifungal agents. The knowledge of major route for human cryptococcal infection (inhalation of infectious particles from saprophytic sources) and a total of 60 *C. neoformans* isolates obtained from AIDS patients with cryptococcal meningitis between October 2001 and April 2002 justify the study of the habitats of these yeasts as probable sources of cryptococcosis in this city.

KEYWORDS: *Cryptococcus neoformans;* Environmental isolates; Pigeon droppings; Susceptibility testing.

INTRODUCTION

C. neoformans is known as an opportunistic human pathogen that causes cryptococcosis, a life-threatening illness that usually manifests as meningoencephalitis mainly in immunocompromised patients[6].

This species has five serotypes (A, B, C, D and AD), and recently was subdivided into three varieties known as *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotypes B and C)[15]. Under this classification the correct designations for serotype AD isolates were not yet resolved[22].

C. neoformans serotypes A and D have been isolated from various sources in nature[25]. Their association with pigeon's excreta is known, but they have also been isolated from droppings of a large variety of avian species, mainly *Psittacidae* birds[5,13]. On the other hand, *C. neoformans* var. *gattii* has been recovered from the environment from plants detritus of some *Eucalyptus* species, such as *Eucalyptus camaldulensis* and *E. tereticornis* and from tropical trees hollow[21,25].

The treatment regimens for cryptococcal meningitis have remained focused on amphotericin B with or without fluocytosine for initial or induction treatment, while azoles (fluconazole or itraconazole) remain the agent of choice for long-term maintenance therapy, to prevent recurrences, or as available therapeutic alternative[29]. Few reports of resistance to amphotericin B, fluconazole or itraconazole have been published concerning *C. neoformans*[10,30]. However, the widespread use of fluconazole for long-term suppressive (maintenance) therapy should be associated with the development of less susceptible strains of this fungus to this antifungal agent in cryptococcal infections[1,4]. The emergence of *C. neoformans* resistance against commonly used antifungal agents[11] has intensified the need of evaluation of *in vitro* susceptibility of this fungus.

The occurrence of cryptococcosis in Goiânia[12] and its importance in immunocompromised patients justify the interest in studying the environmental sources of *Cryptococcus* species in this city localized in the Brazilian midwest region. In this work, we proposed to verify the presence of these yeasts in urban saprophytic sources and evaluate the varieties, the serotypes and the *in vitro* susceptibility of *C. neoformans* isolates to fluconazole, itraconazole and amphotericin B.

MATERIAL AND METHODS

Sampling: Samples of pigeon and the avicultures avian droppings, soil, ornamental trees hollow and samples of barks, leaves, seeds of

(1) Instituto de Patologia Tropical e Saúde Pública da Universidade Federal de Goiás, Goiânia, Brazil.
(2) Hospital de Urgências de Goiânia, Goiás, Brazil.
Correspondence to: Dra. Maria do Rosário Rodrigues Silva, Instituto de Patologia Tropical e Saúde Pública, Rua Delenda Rezende de Melo esq. com 1ª Avenida. Setor Universitário, Caixa Postal 131, 74605-050 Goiânia, GO, Brasil. Phone: (55-62) 209-6127. Fax: (55-62) 5211839. E-mail: rosario@iptsp.ufg.br

the genus *Eucalyptus* trees, as well as plants detritus from the ground were collected in 35 different places in Goiânia, Goiás State, Brazil, at least twice over a period of one year (2002-2003). The samples were collected in protected from direct sunlight and unprotected places. The number and the type of environmental samples collected at different places are shown in Table 1.

Environmental samples from pigeon and other avian droppings and from soil were collected with spatulas and placed in identified clean plastic bags. The samples of trees hollow were collected from discolored fibrous wood in advanced stages of decay in the inner side of the hollows. *Eucalyptus* debris (barks, leaves and seeds) were collected under the trees canopies by hand and by using spatulas.

Isolation and sample processing: The samples were processed by according CASALI *et al.*[7] in 48 hours or stored at 4 - 6 ºC until processing. About 1.0 g of weathered avian droppings and soil or 2.5 g of material obtained from trees were added to 9.0 mL of sterilized saline solution 0.9% with both chloramphenicol and ampicillin at 200 mg L^{-1} each and shaken vigorously for five minutes in Vortex type agitator. The mixture was left at room temperature for about 10 - 15 minutes. Next, ten-fold serial dilutions were made and aliquots of 10^{-1} and 10^{-2} from the supernatant (birds excretas and soil) or of 10^{-3} and 10^{-4} (material of trees) were streaked onto duplicate plates containing bird seed (*Guizotia abyssinica*) and Sabouraud dextrose agar. The cultures were incubated at 32 ºC and at room temperature for 21 days. Brown yeast-like colonies of suspected *C. neoformans,* detected on bird seed agar, were subcultured to obtain single colonies on Sabouraud dextrose agar plate.

Identification: All environmental isolates were identified by colony morphology, microscopic morphology of yeast cells and by carbohydrate assimilation tests. *C. neoformans* isolates were further identified on the basis of melanin synthesis on bird seed agar, presence of a capsule on India ink preparation, urease production on Christensen medium and the ability to growth at 37 ºC[18].

Biotyping and serotyping of *C. neoformans:* The determination of *C. neoformans* variety was performed on canavanine-glicyne-bromothymol blue (CGB) medium[20] and the serotypes were determined by the slide agglutination test using the Crypto Check Iatron RM 304-K kit (Iatron Labs., Tokyo, Japan) according to the manufacturers' instructions.

Antifungal susceptibility testing: Standard broth microdilution method recomended by the NCCLS M27-A2[26] was performed to all *C. neoformans* isolates. Amphotericin B (Squibb, Princeton, NJ, USA) and itraconazole (Jansen Pharmaceuticals, Beerse, Belgium) were dissolved in dimethyl sulfoxide and fluconazole (Pfizer International, New York, USA) was dissolved in distilled water. Further dilutions of each antifungal agent were prepared with RPMI 1640 medium (Sigma) containing L-glutamine, without sodium bicarbonate and buffered to a pH 7.0 with 0.165M MOPS (morpholinepropanesulfonic acid, Sigma).

The suspension of yeast from 48-h-old cultures was prepared in sterile saline (0.85%), adjusted with a spectrophotometer to a cell density of a 0.5 McFarland standard at a wavelength of 530 nm. This suspension was diluted at 1:50 followed by a 1:20 dilution in RPMI 1640 in order to obtain a final concentration of 1 x 10^3 to 5 x 10^3 CFU/mL. Prior to antifungal susceptibility testing, each isolate was subcultured at least twice on Sabouraud dextrose agar to ensure purity and optimal growth.

Microtitre plates were covered with 100 µL of the different concentrations of the antifungals agents and added with 100 µL of the yeast suspension to obtain a final inoculum of 0.5 x 10^3 to 2.5 x 10^3 CFU/mL and the final concentrations of the antifungal agents ranging from 0.03 to 64 µg/mL for fluconazole and 0.007 to 16 µg/mL for itraconazole and amphotericin B. The microdilution trays were incubated at 35 ºC and read after 72 hours. All susceptibility tests were performed twice by each antifungal agent. One standard strain, *C. parapsilosis* ATCC 22019, was used as internal control in each run of test[26].

Reading results: The MIC end points were defined for amphotericin B as the lowest concentration of drug which resulted in a complete inhibition of visible growth. The MICs for the two azoles were defined as the lowest concentration of drug that produced a prominent decrease in fungal growth compared to that one of drug-free growth control.

Interpretation of results: Resistance for *C. neoformans* environmental isolates was defined as: MIC ≥ 64 µg/mL for fluconazole, MIC ≥ 1 µg/mL for itraconazole and MIC ≥ 2 µg/mL for amphotericin B according to NGUYEN & YU (1998)[27] and LOZANO-CHIU *et al.* (1998)[23].

Table 1
Number of environmental samples collected from several materials at different places in Goiás, Goiânia, Brazil

Places		Pigeon droppings	Other avian excretas	Soil	*Eucalyptus*	Ornamental trees	Total
Residential buildings	(2)	06	-	-	-	-	06
Squares and streets	(15)	49	-	04	08	22	83
Hospitals	(3)	26	-	08	18	-	52
Churches	(6)	92	-	-	-	-	92
Nutritious industry	(1)	04	-	-	-	-	04
Avicultures	(7)	-	44	-	-	-	44
Police Academy	(1)	-	-	-	09	-	09
Total		177	44	12	35	22	290

( ) number of places analyzed

Table 2
Cryptococcus species other than *neoformans* isolated from 290 environmental samples collected in the urban environment of Goiânia, Goiás, Brazil

Cryptococcus species	Places							
	Residential building	Square and street	Hospital	Church	Nutritious industry	Aviary	Police Academy	Total
C. albidus	01	07	09	39	04	02	-	62
C. laurentii	-	03	02	05	01	-	-	11
C. uniguttulatus	-	-	01	07	-	02	-	10
C. luteolus	-	-	02	01	-	-	-	03
C. gastricus	-	-	01	-	01	01	-	03
C. terreus	-	-	01	-	-	-	-	01

RESULTS

Out of 290 environmental samples collected, forty-one *C. neoformans* isolates were obtained from pigeon droppings and material associated with eucalyptus trees. No *C. neoformans* was discovered from avicultures avian droppings, soil without excretas and ornamental trees hollow.

All isolates of *C. neoformans* belonged to *C. neoformans* var. *grubii* (serotype A). *C. neoformans* var. *gattii* has not been isolated from any of the samples examined. *C. albidus*, *C. laurentii* and other species of *Cryptococcus* were isolated from sites within the urban perimeter, including the area containing pigeon droppings (Table 2).

Out of the 177 samples of pigeon droppings, collected in 22 places, *C. neoformans* var. *grubii* was recovered from 36 specimens (20.3%) from five sites (22.7%). These positive sites corresponded to three churches, one hospital and one nutritious mass industry. *C. neoformans* var. *grubii* was isolated in 35 pigeon dropping samples collected in nine protected places and in one specimen collected in 13 unprotected places (Table 3).

Table 3
Distribution of *Cryptococcus neoformans* var. *grubii* isolated from pigeon droppings in different places

Description	Place	N	Samples	
			n	(%)
Churches	P	82	34	(41.5)
	UP	10	-	-
Hospital	P	-	-	-
	UP	26	01	(3.8)
Squares and streets	P	08	-	-
	UP	41	-	-
Nutritious industry	P	04	01	(25)
	UP	-	-	-
Residential buildings	P	06	-	-
	UP	-	-	-
Total	P	100	35	(35.0)
	UP	77	01	(1.3)

P = protected place; UP = unprotected place; N = total number; n = number of positive samples.

C. neoformans var. *grubii* was recovered only in five barks samples (14.3%) of the genus *Eucalyptus* trees collected from one hospital and from one Police Academy.

None of the environmental isolates of *C. neoformans* showed *in vitro* resistance to three antifungal agents tested. MIC ranges for the *C. neoformans* var. *grubii* isolates were between 0.25 to 2.0 µg/mL for fluconazole, 0.007 to 0.12 µg/mL for itraconazole and 0.015 to 0.12 µg/mL for amphotericin B. There was no difference between MIC_{90} values for itraconazole and amphotericin B (0.12 µg/mL) while for fluconazole the MIC_{90} was 1.0 µg/mL. MICs for the quality control strain were within the expected limits for all antifungal agents tested.

DISCUSSION

Air transmission route of the desiccated yeast cells or basidiospores present in the environment which could have the function of *C. neoformans* dissemination focus justifies the research of saprophytic sources of this agent. The positive samples of *C. neoformans* var. *grubii* observed in our study were localized in places close to the great flow of people, suggesting a high exposure to the fungus. The occasional exposure to isolates of *C. neoformans* could be associated with the infection risk in a given population[6]. In Goiânia, FERNANDES *et al.* (2003)[12] related a large number of cases (60 cases) of cryptococcosis in patients with AIDS during a six months period. The appearance of *C. neoformans* serotype A as the most prevalent agent of this disease in Goiânia, confirms the exposure to environmental sources of this variety.

Avian droppings have been related as important substrates for the presence and maintenance of yeast in nature[13]. The churches studied, sites where a large amount of pigeon droppings was verified, presented a heavy contamination by *C. neoformans*. This contamination was higher in protected places than in unprotected places. This can be explained by the high resistance of *C. neoformans* to desiccation, which favors the survival of the fungus when competing with other microbial populations[19].

No evidence of isolation of this fungus in avian droppings from avicultures probably was due to collection of a little weathered samples, since wet droppings undergo the bacterial decomposition, causing a strong alkalinization on the substrate and growth inhibition of *C. neoformans*[19].

With respect to the other samples collected, the absence of positivity of the *C. neoformans* could have occurred because of the overgrowth of fast-growing filamentous fungi. This possibly led to false-negative results in the bird seed agar plating method[14].

In spite of a high correlation of *C. neoformans* var. *gattii* with eucalyptus, we have not found this variety. In Brazil, MONTENEGRO & PAULA (2000)[25] have recovered *C. neoformans* var. *gattii* from *Eucalyptus* spp trees vegetable material in São Paulo. This variety was also detected in Teresina, Brazil, in eucalyptus samples, but its isolation happened after inoculation in mice[3]. The biologic competition among microorganisms in wood decomposition process explains the difficulty to isolate these fungi[16]. Therefore, furthermore extensive studies are needed to confirm the presence or absence of *C. neoformans* var. *gattii* from environmental sources in Goiânia.

The presence of *C. albidus*, *C. laurentii* and *C. uniguttulatus* recovered in the environment is an important data. These species have been reported as agents of opportunistic infection such as meningitis, lung infections, fungaemia, abscess and skin infection, mainly in patients with great deterioration of the immune response[8,17].

The low MICs values of *C. neoformans* environmental isolates presented to fluconazole, amphotericin B and itraconazole are consistent with the current literature on which the majority of the *C. neoformans* isolates are *in vitro* susceptible to these three antifungal[1,9]. However, low sensivity of environmental isolates to fluconazole has been demonstrated. BARONI (2001)[2], using the Etest methodology, verified the resistance to this antifungal agent for 75.86% of *C. neoformans* isolates obtained from pigeon droppings in Rio de Janeiro.

Resistance of *C. neoformans* clinical isolates to fluconazole has been related in AIDS patients and in immunocompetent patient[10,24]. The isolate resistance to this antifungal agent can occur due to a previous or extended maintenance treatment, or prophylaxis in immunocompromised patients[30]. However, fluconazole-resistant *C. neoformans* isolates obtained from immunocompetent patient without exposure to this azole suggest that environmental strains can be primarily resistant to this antifungal agent[28].

The present study showed the presence of *C. neoformans* in urban environmental sources at places of high public circulation in Goiânia. Since the exposure to dry inhalable spores from these reservoirs seems to be linked to cryptococcal infection and due to the immunocompromised individuals are considered the patients at risk for cryptococcosis, the elucidation of the ecology of this yeast has been very important.

RESUMO

Caracterização de *Cryptococcus neoformans* isolados de fontes ambientais urbanas na cidade de Goiânia, estado de Goiás, Brasil

Cryptococcus neoformans é um fungo patogênico oportunista que causa meningoencefalite como a apresentação clínica mais importante em pacientes imunocomprometidos, principalmente, em pessoas infectadas pelo HIV. O agente é uma levedura encapsulada, comumente encontrada em solo enriquecido com excretas de aves e em resíduos de plantas. O total de 290 amostras de excretas de pombos e outras aves, de árvores ornamentais e materiais vegetais de *Eucalyptus* foram coletadas para estudar possíveis fontes ambientais de *Cryptococcus* spp, na cidade de Goiânia, Goiás. A determinação das variedades, sorotipos e suscetibilidade *in vitr*o frente a fluconazol, itraconazol e anfotericina B dos isolados de *C. neoformans* foram realizadas. *C. neoformans* var. *grubii* (sorotipo A) foi a única isolada, ocorrendo em 36 (20.3%) das 177 amostras fecais de pombos e em 5 (14.3%) das 35 amostras de *Eucalyptus*. Nenhum dos isolados ambientais de *C. neoformans* mostrou resistência *in vitro* aos três antifúngicos avaliados. O conhecimento da principal via para infecção criptocócica humana, isto é inalação de partículas infecciosas de fontes saprofíticas e a ocorrência de 60 casos de criptococose em pacientes com AIDS, em Goiânia, entre outubro de 2001 e abril de 2002, justificam o estudo de habitats do agente como prováveis fontes de criptococose nesta cidade.

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