

Prevalence of Decreased Susceptibility of *Cryptococcus* spp. Isolates to Fluconazole in Urban Sources of Presidente Prudente

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Abstract: The genus *Cryptococcus* is composed of encapsulated yeasts that have the ability to infect and cause disease in humans, as *Cryptococcus gattii*/*Cryptococcus neoformans* species complex. Facing the current research panel, few studies about cryptococcosis and epidemiological data have been carried out in the western region of São Paulo state. This study aimed to verify the occurrence of *Cryptococcus* spp. in public areas of the city of Presidente Prudente, using pigeon droppings and tree hollows as an environmental source. The samples were identified by conventional mycological methods. Were collected 54 samples from pigeon droppings and 62 samples from tree hollows of the genus *Licania*. And of these samples, 47 (87.0%) and 14 (22.5%) had positive growth of yeast from pigeon droppings and tree hollows, respectively. *Cryptococcus* was identified in 11 (20.4%) of the samples from pigeon droppings and 3 (21.4%) of those from tree hollows. The following species were identified: *Cryptococcus neoformans* (7.15%), *Cryptococcus laurentii* (14.28%), and *Cryptococcus albidus* (78.57%). Decreased susceptibility to fluconazole was observed for some isolates. Fluconazole exhibited a limited *in vitro* activity, particularly against *Cryptococcus albidus* and *Cryptococcus laurentii*. Identification and susceptibility testing of *Cryptococcus* spp. should be performed on a routine basis in view of their unpredictable susceptibility profiles.

Key words: Droppings, pigeons (*Columba livia*), tree hollows, *in vitro* *Cryptococcus* spp., susceptibility test.

1. Introduction

Cryptococcus (*C.*) spp. is a genus of fungi belonging to class basidiomycota, which in the asexual phase occur as yeasts from 2-10 µm diameters. Pathogenic species are surrounded by a polysaccharide capsule, which is their principle morphological characteristic [1]. Although there are more than 100 species of *Cryptococcus*, the responsible for the vast majority of human infections is the *Cryptococcus gattii*/*Cryptococcus neoformans* species complex [2-4].

C. neoformans is composed of a polysaccharide capsule with polymers of xylose, manose and gluconic

acid, increasing its virulence. Beyond this, it has the ability to neutralize host defense cells by means of substances such as melanin and mannitol, which destabilize the host cell membranes through phospholipases. It has the ability to grow at a temperature of 37 °C and is characterized as a cosmopolitan fungus, present in regions with tropical, subtropical and temperate climates [5-8]. Within this ecological niches are aged and dried bird feces, principally from pigeons (*Columbia livia*), and there is a lower concentration of bacteria, and therefore less ecological competition. Furthermore, these substrates are rich in urea and creatine, substrates that favor the growth of yeast [8]. The isolation of *C. neoformans* from soil contaminated with pigeon excrement, in which the fungus remains viable, is the basis of the

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concept that the infection has an environmental origin [9, 10].

C. gattii occurs principally in the regions with a tropical, subtropical climate, as well as in regions with the lesser extent temperate. In Brazil, it was identified in the Parque do Ibirapuera, located in Sao Paulo, and in eucalyptus plantations in Teresina, Piaui state. However, studies showed the occurrence of *C. gattii* in tropical trees from north and northeast of Brazil, such as *Cássia* (*Cassia spectabilis*), *Oiti* (*Licania tomentosa*), *Fícus* (*Ficus benjamina*), *Mulungu* (*Erythrina verna*) and *Guettarda* (*Guettarda viburnoides*) [11-14].

In addition to *C. neoformans*/*C. gattii* complex, there are other species of non-*C. neoformans* and non-*C. gattii* considered saprophytes, and non-pathogenic. Those representative species, *C. albidus* and *C. laurentii*, have been isolated from food, aquatic environments, soil, bird droppings and human skin [15]. However, in the last years it was verified that these non-pathogenic species can cause injuries to individuals with some types of immune deficiency, compromising the central nervous system leading to fungemia [16-18].

This paper evaluated the occurrence of *Cryptococcus* spp. In public areas of Presidente Prudente city, in Sao Paulo state, and determination the prevalence of *Cryptococcus* spp. isolated from pigeon droppings found in locations such as Praça Monsenhor Sarrion, Catedral São Sebastião, Universidade do Oeste Paulista—UNOESTE campus, and outdoor areas of the Hospital Regional de Presidente Prudente—HR. Furthermore, the study determined the prevalence of *Cryptococcus* spp. within tree hollows of *Licania tomentosa*, located in the areas where pigeon dropping isolates were collected, and the MIC (minimum inhibitory concentration) of the fungicide fluconazole was determined for species of *Cryptococcus* isolated from these areas. The tree species was chosen due to its high prevalence in locals of collect pigeon dropping.

2. Materials and Methods

The city of Presidente Prudente is located in the western region of Sao Paulo state, 560 km from the capital, at latitude 22°07'04" S, longitude 51°22'57" W. This city is part of a transitional climate area, with elevated temperature and precipitation—150 to 200 mm per month—in spring and summer, with a maximum mean temperature between 27 °C and 29 °C and extratropical systems in the autumn and winter with low humidity and precipitation—20 to 50 mm per month—and mean minimum temperatures between 16 °C and 18 °C [19].

2.1 Sample Collection

A total of 116 samples of bird droppings were collected from different locations in the City of Presidente Prudente, SP, during late Autumn until Spring (from May 1st to November 20th), 2014. The specimens were placed in the sterile tube collectors, standardized, and sent to the Microbiologia and Imunologia building, Bloco G, campus I of the Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, State of São Paulo, Brazil.

For the collection of samples from bird droppings and tree hollows, ten sampling sites were defined in Praça Nove de Julho, Praça Monsenhor Sarrion—Catedral São Sebastião, Hospital Regional de Presidente Prudente—HR, and Campus I of Universidade do Oeste Paulista (UNOESTE).

2.2 Isolation and Identification of the Yeast

The collection of pigeon dropping samples was carried out by means of sterile spatulas and placed in sterile plastic pots identified by the date and location of collection. The samples from tree hollows were obtained with sterile swabs by passing it inside the tree hollows and transferring them to labeled sterile plastic pots. To isolate *Cryptococcus* spp., 1 g of the biological material was weighed and placed into Falcon tubes containing 10 mL of sterilized saline solution (0.85%

NaCl) supplemented with 0.05 g/L chloramphenicol. The specimens were subsequently stirred for three minutes in a vortex apparatus. After stirring, the materials were allowed to stand for five minutes and were then diluted with saline containing chloramphenicol (1:100 dilution). A 0.1 mL aliquot of the supernatant was removed and seeded in triplicate on Niger agar. The cultures were incubated at room temperature and observed daily for up to 10 days to evaluate the colony morphology.

To identify *Cryptococcus* species, isolates were subjected to morphological and physiological tests, including the production of phenol oxidase on Niger agar, the detection of urease in Christensen's media, carbon and nitrogen assimilation. For the biochemical tests, the auxanographic technique was used to evaluate the assimilation of 11 carbon sources (dextrose, lactose, maltose, sucrose, inositol, galactose, cellobiose, dulcitol, melibiose, trehalose, and raffinose) and two nitrogen sources (peptone and potassium nitrate).

2.3 Susceptibility Testing

The susceptibility to fluconazole test was performed following the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (EUCAST) [20]. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains. The antifungal agent used for the study was fluconazole (Sigma S. A.), the MIC end point was defined as 50% of growth inhibition.

3. Results

3.1 Environmental Isolates

A total of 54 samples were collected from pigeon droppings and 62 samples from tree hollows. The frequency of yeast isolation was 87.0% (n = 47) in pigeon droppings and 22.5% (n = 14) in tree hollows. *Cryptococcus* was identified in 23.4% (n = 11) samples isolated from pigeon droppings and in 21.4% (n = 3) samples from tree hollows (*Licania* sp).

The samples of *Cryptococcus* spp. were evaluated as pigeon droppings collected either from cement, dirt or tree hollows. Out of 32 samples of pigeon droppings collected from cement, 5 (15.6%) were positive for *Cryptococcus* spp., and out of a total of 22 samples collected from dirt, 6 (27.3%) were identified as *Cryptococcus* spp.

By location of collection, at the "Praça Monsenhor Sarrion", 6 out of 26 (23.1%) positive samples for *Cryptococcus* spp., at "Praça Nove de Julho", 4 out of 16 samples (25.0%) were positive for *Cryptococcus* spp. and at Unoeste Campus I, 1 out of 12 samples (8.3%) was positive for *Cryptococcus*. "Hospital Regional de Presidente Prudente", did not presented positive results. Additionally, 4 (7.4%) samples were positive for *Sacharomyces* spp., 9 (16.6%) for *Candida* spp., 16 (29.6%) for *Rhodotorula* spp. and 7 (13%) presented growth of *Trichosporon* spp. About samples from tree hollows, 8 (12.9%) samples had *Rhodotorula* spp. and 3 (4.8%) *Trichosporon* spp. Isolated, as shown in Table 1.

About *Cryptococcus* spp. positivity in droppings, 8 (78.6%) were identified as *C. albidus*, 2 (14.3%) as *C. laurentii* and 1 (7.1%) as *C. neoformans*, shown in Table 2. From the 3 tree hollow *Licania tomentosa* samples, positive for *Cryptococcus*, 100% were identified as *C. albidus* and were isolated from "Praça Monsenhor Sarrion".

3.2 Susceptibility Testing

Decreased susceptibility to fluconazole was observed for some isolates. The percentage of strains with decreased *in vitro* antifungal susceptibility included a total of 9 strains (82.0%, 9/11) of *C. albidus* which were found to have an MIC to fluconazole ≥ 16 mg/L, and 1 strain (50.0%, 1/2) of the *C. laurentii* strains was found to have a fluconazole MIC of ≥ 16 mg/L (Table 2).

4. Discussion

Since the 1950s, through the research developed by

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Table 1 Collection sites, number of samples, ambient temperature, environmental niches of city and region, and absolute / relative isolation of *Cryptococcus* in Presidente Prudente, State of São Paulo, Brazil.

Collections sites	Number os samples collected/positive sample	Environmental niche	Isolate	Average ambient temperature (°C)	Absolute isolation (%) /relative isolation (%)
Praça Monsenhor Sarrion	26/06		<i>Cryptococcus</i> spp.	22.48	11.1/23.1
	26/02		<i>Candida</i> spp.		3.7/7.7
	26/07		<i>Rhodotorula</i> spp.		12.9/26.9
	26/02		<i>Trichosporon</i> spp.		3.7/7.7
Praça 09 de julho	16/04	<i>Columbia livia</i> dropping	<i>Cryptococcus</i> spp.	22.09	7.5/25.0
	16/04		<i>Candida</i> spp.		7.5/25.0
	16/07		<i>Rhodotorula</i> spp.		12.9/43.7
	16/05		<i>Trichosporon</i> spp.		9.2/31.2
	16/01		<i>Saccharomyces</i> spp.		1.9/6.2
Universidade do Oeste Paulista (UNOESTE)*	12/01		<i>Cryptococcus</i> spp.	20.42	1.9/8.3
	12/03		<i>Candida</i> spp.		5.5/25.0
	12/02		<i>Rhodotorula</i> spp.		3.7/16.6
	12/03		<i>Saccharomyces</i> spp.		5.5/25.0
Total	54/47				87.0/-
Praça Monsenhor Sarrion	26/03	Tree hollows (Licania tomentosa)	<i>Cryptococcus</i> spp.	18.9	4.8/11.5
	26/06		<i>Rhodotorula</i> spp.		9.6/23.0
	26/02		<i>Trichosporon</i> spp.		3.3/7.7
Praça 09 de julho	09/02		<i>Rhodotorula</i> spp.	21.5	3.3/22.2
	09/01		<i>Trichosporon</i> spp.		1.6/11.1
Regional hospital*	27/00	-	-	-	-
Total	62/14				22.5/-
Samples total	116/61				52.5/-

* At the Universidade do Oeste Paulista (UNOESTE) no genus of the tree *Licania tomentosa* was found. At the Regional hospital* no *Columbia livia* droppings were found.

Table 2 MIC values of environmental isolates of *Cryptococcus* spp. to fluconazole.

Species (%)	Environmental niche (Number of isolates)	Collections sites	Average ambient temperature (°C)	Median (mg/L)	Mode (mg/L)	MIC (mg/L) range
<i>Cryptococcus neoformans</i> (7.2)	<i>Columbia livia</i> droppings (01)	Universidade do Oeste Paulista (UNOESTE)	22.1	1.0	-	1.0
<i>Cryptococcus albidus</i> (78.6)	<i>Columbia livia</i> droppings (08)	Praça Monsenhor Sarrion/Praça Nove de Julho	24.4	64.0	64.0	0.5-64.0
	Tree hollows (<i>Licania tomentosa</i>)(03)	Praça Monsenhor Sarrion	18.9			
<i>Cryptococcus laurentii</i> (14.2)	<i>Columbia livia</i> droppings (02)	Praça Nove de Julho	22.5	24.0	-	16.0-64.0
Total	14	-	21.9			0.5-64.0

[9, 10], the presence of *Cryptococcus* spp. is related to pigeon droppings, which establishes the environmental origin of infection by this fungus. Similar study, Ref. [21] during winter identified 14 samples (7.68%) positive for *Cryptococcus* spp. in pigeon droppings, a lower result than in the present study. This difference in prevalence could be due to the bad growth conditions

for fungus, possibly because of lower temperatures when the samples were collected [21]. Larger proportion of isolates identified as *Cryptococcus* spp. at Praça Nove de Julho is possibly related to the large quantity of droppings present there, whereas at Unoeste campus I, where is daily cleaning, and there was no accumulation of droppings, making their isolation

more difficult.

The *Cryptococcus* species known as non-*C. neoformans*—*C. laurentii* and *C. albidus* were isolated with a higher frequency compared to the only sample identified as *C. neoformans*. These yeasts are found in air, water, wood, soil, pigeon droppings, and in foods such as fruit, pork and beans [18]. *C. laurentii* is distinguished by its adaptive ability to live in various environments, as it is isolated from areas with tropical climates as well as regions with below zero temperatures, such as Antarctica [22]. Ref. [23] identified 23 samples of *Cryptococcus neoformans* in pigeon droppings and 5 samples of *C. neoformans* in decomposing eucalyptus leaves. Additionally, a study carried out by Ref. [24] with bird droppings of captive birds and bird droppings in the external environments identified 38 samples of *Cryptococcus laurentii*, showing a heterogeneity of the isolates.

Furthermore, starting with the droppings contaminated with *Cryptococcus* it is possible for a spread and colonization of nearby tree hollows by these yeasts [25]. This fact was observed in our study, since at Praça Monsenhor Sarrion 6 (75.0%) *C. albidus* out of 8 samples were isolated from this square, the same place where 3 (100%) of the samples of *C. albidus* were isolated from tree hollows. Ref. [26] studied species of trees characteristic of the region and local flora in Spain, they identified 56 *Cryptococcus* spp., 31 (55.4%) of this were *C. albidus*, 14 (25%) *C. gattii*, 9 (16.1%) *C. neoformans* and 2 (3.6%) *C. laurentii*. Their results show the high prevalence of *C. albidus* isolates as well as evidence that *C. gattii* is not the most frequently isolated type in these locations [26].

The present study corroborates to Ref. [28], who identified *Cryptococcus* spp. in 5 samples of tree hollows and bark, 2 of *C. neoformans*, 2 of *C. gattii* and one of *C. laurentii*, in Vitória, Espírito Santo, Brazil. As presented 3 positive *Cryptococcus* spp. isolated from tree hollows, thus 100.0% of samples are positive for *C. albidus* in tree hollows (*Licania tomentosa*) at Praça Monsenhor Sarrion, where large amounts of

droppings and pigeons were seen.

We did not observe climatic influences over the *Cryptococcus* isolates (Average of 21.9 °C), a greater number of positive samples might demonstrate a controversy result. Other studies found *C. albidus*, *C. laurentii* and *C. neoformans* from pigeon droppings and plant material with different prevalence according to the climate, region and quantity of material collected [13, 27, 29, 30].

Our study also identified other yeasts in pigeon droppings different than *Cryptococcus*, such as *Rhodotorula* spp. in the highest frequency, followed by *Candida* spp., *Trichosporon* spp., and *Saccharomyces* spp. Yeasts of genus *Rhodotorula* and *Trichosporon* were also found in tree hollows. These results are similar to reported by Ref. [31] that evaluated pigeon droppings and tree hollows, where in 13 *Cryptococcus* spp. positive samples (10 *C. neoformans* and 3 *C. laurentii*), 9 *Candida albicans*, 1 *Candida tropicalis*, 1 *Candida krusei*, 1 *Candida parapsilosis*, 6 *Rhodotorula* spp. and 3 *Trichosporon* were found. Authors isolated *Candida glabrata* in tree hollows. These data are similar to those found in our work, which confirms that different types of yeasts can share the same ecological niche.

Our results and data from the literature indicate that *C. albidus* and *C. laurentii* exhibited a decreased susceptibility to fluconazole, according to those shown by Ref. [16]. It is important to remember that the isolates studied by Ref. [16] were clinical isolates, which showed the same susceptibility profiles as the present study.

Studies have linked resistance to azole antifungals, particularly fluconazole in large scale fungicide use in agriculture, which easily disperse in the air, making previously susceptible environmental strains resistant [32, 33]. It has also been related to an intrinsic characteristic of *Cryptococcus*, since studies with clinical and environmental samples 20 years before the beginning of the use of fluconazole had already shown resistant strains. This intrinsic characteristic is

considered an adaptive response to stress, and as during the use of fluconazole, it could be reversible with the cessation of use. The greatest impact of this is that fewer strains are susceptible [34]. In view of the different susceptibility profiles of fungal species, and large distribution of these species in the environment, the characterization at species level of clinical isolates of the *Cryptococcus* is compulsory. In addition, the identification of these fungi can be difficult for clinical laboratories, making it necessary to dispatch isolates of *Cryptococcus* spp. to reference centers. Since such identification could take several weeks, *in vitro* susceptibility testing of isolates belonging with genus *Cryptococcus* should be carried out in clinical laboratories on a routine basis.

5. Conclusions

In this study pathogenic species had been less isolated (1 sample of *C. neoformans*) in pigeon droppings and tree hollows (*Licania tomentosa*), than other species such as *C. albidus* and *C. laurentii* had been isolated in greater number, and exhibited a decreased susceptibility to fluconazole.

The identification of infested areas, proper control of birds, and the disinfection of these environments are essential for the epidemiological control of cryptococcosis. Identification and susceptibility testing of *Cryptococcus* spp. should be performed on a routine basis in view of their unpredictable susceptibility profiles.

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