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*Original Article*

# Isolation of *Cryptococcus* species from the external environments of hospital and academic areas

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## Abstract

Introduction*:* Fungi of the genus *Cryptococcus* are cosmopolitan and may be agents of opportunistic mycoses in immunocompromised and sometimes immunocompetent individuals. *Cryptococcus* species are frequently isolated from trees and bird excreta in the environment and infection occurs by inhalation of propagules dispersed in the air. The aim was to investigate *Cryptococcus* species in bird excreta and tree hollows located in a university hospital area and in an academic area of a university campus.

Methodology: A total of 40 samples of bird excreta and 41 samples of tree hollows were collected. The identification of the isolates was done by classical methodology and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Results: Twenty (62.5%) isolates of *Cryptococcus* were found in bird excreta and 12 (37.5%) in tree hollows. *C. laurentii* (currently *Papiliotrema laurentii*) was the most frequent species in both samples, being found in 5 samples of excreta and in 8 tree hollows. The diversity of species found in excreta (*C. laurentii*, *C. albidus* [currently *Naganishia albida*], *C. liquefaciens* [currently *N. liquefaciens*], *C. friedmanii* [currently *N. friedmannii*] and others) was higher than in tree hollows (*C. laurentii*, *C. flavescens* [currently *Papiliotrema flavescens*], and other yeasts).

Conclusion: Many *Cryptococcus* species were isolated from excreta and tree hollows, and this fact is important for understanding the environmental epidemiology of those emerging pathogens for public health, as a way to implement surveillance actions and control of cryptococcosis.

**Key words:** *Cryptococcus* species; environmental health; opportunistic infection.

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## Introduction

The fungi of the genus *Cryptococcus* are found in the environment, widely distributed in nature around the world, and some species are of great importance as pathogens for humans and animals. The propagules of these fungi can be inhaled and cause lung and neurological infections, which can be fatal [1]. Infections caused by *Cryptococcus* species, called cryptococcosis, are among the most important opportunistic fungal diseases affecting man [2].

The main etiological agents of cryptococcosis correspond to two species complexes: *C. neoformans sensu lato* and *C. gattii sensu lato* [3,4]. Cryptococcosis is one of the main opportunistic infections that primarily affect not only patients with acquired immunodeficiency syndrome (AIDS) but also individuals with other conditions that lead to immunosuppression. However, the disease can also occur in immunocompetent individuals, especially when caused by *C. gattii sensu lato* There is great interest in the agents and the disease in the world public health, especially because of the severity and forms of infection that occurs in the environment [5,6].

The ecological niche of the different species of *Cryptococcus* is associated with excreta of birds and decomposing plants [7]. *C. neoformans sensu lato* has

been isolated from the habitat of birds and their dry excreta that are present in different environments, both urban and rural. It has a wide geographic distribution and is found in all Brazilian regions as well as all the countries of the world [8,9,10]. However, there are reports of isolations from tree species such as *Eucalyptus camaldulensis*, *Olea europaea*, *Pinus* and *Acacia Amarillo* [11,12]. *C. neoformans sensu lato* causes infections mainly in immunocompromised patients [2].

*C. gattii sensu lato* is more related with organic matter that is present in trees and decomposing wood. It has great geographical distribution in many countries in the tropical and subtropical areas of the world [13]. *C. gattii sensu lato* is still considered a primary pathogen, a life-threatening agent, and most cases of infection occur in the northeastern region of Brazil [14,15,16].

Other species of *Cryptococcus* are considered potentially pathogenic, such as *C. laurentii* (currently *Papiliotrema laurentii*), *C. albidus* (currently *Naganishia albida*), and *C. flavescens* (currently *P. flavescens*), which have been reported in some studies [6,7,17,18,19]. These species are also isolated from the environment in organic matter, such as tree debris and bird excreta [7,17,19]. Andrade-Silva *et al*. [6] and Ferreira-Paim *et al*. [20] reported *Cryptococcus* spp., including *C. laurentii* and *C. neoformans sensu lato*, isolated from dried excreta of birds in domestic environments and in areas around the hospital.

The birds of the urban area are reservoirs of infectious agents that have public health relevance, since the excreta of these birds are shelters for species of *Cryptococcus* [21]. Pigeons (*Columba livia*) are animals that reach the urban environment and feed on leftover food lying on the ground. This condition favors the presence and reproduction of those birds in the urban environment, multiplying the number of reservoirs capable of acting in the chain of transmission of pathogens for human and animals.

Hospital and academic areas are usually frequented by immunocompetent and immunocompromising individuals. Public places that are frequented by pigeons denote the necessity of educational and preventive measures that may contribute to the reduction or elimination of sources of disease agents, such as those caused by opportunistic fungi [22].

*Cryptococcus* spp. that are traditionally considered to be pathogenic have medical and sanitary importance, so their presence in the environment contributes to the recognition of the main ecological niches. Also, it is imperative to preventive and control measures, both the environmental dissemination of pathogens (*C. neoformans sensu lato*) and the population of pigeons or other birds. In this context, this study was proposed to investigate species of *Cryptococcus* from bird excreta and tree hollows located in an area of a university hospital and in academic areas of a university campus as well as to discuss the results from a public health perspective. Moreover, this study highlights the importance of plants (and plant detritus) and organic materials (bird excreta) as ecological niches and natural reservoirs of *Cryptococcus* spp. in the environment.

## Methodology

*Sampling sites*

Samples of tree hollows and bird excreta were collected from August to December 2016 in an area that covers about 250 meters around the Clinical Hospital of Uberlandia, which is attached to the Umuarama *Campus* of the Federal University of Uberlandia, in the city of Uberlandia, State of Minas Gerais, Brazil, as shown in Figure 1.

The Clinical Hospital of the Federal University of Uberlandia is a university hospital of high complexity and reference for a neighbor population that includes more than 2 million inhabitants in 86 different municipalities of the Triangulo Mineiro region in Minas Gerais, Brazil. It is the largest provider of services by the State's Unified Health System and the third largest university hospital in Brazil. It has a total of 520 beds and offers urgent, emergency, outpatient, surgical, inpatient, and oncological care.

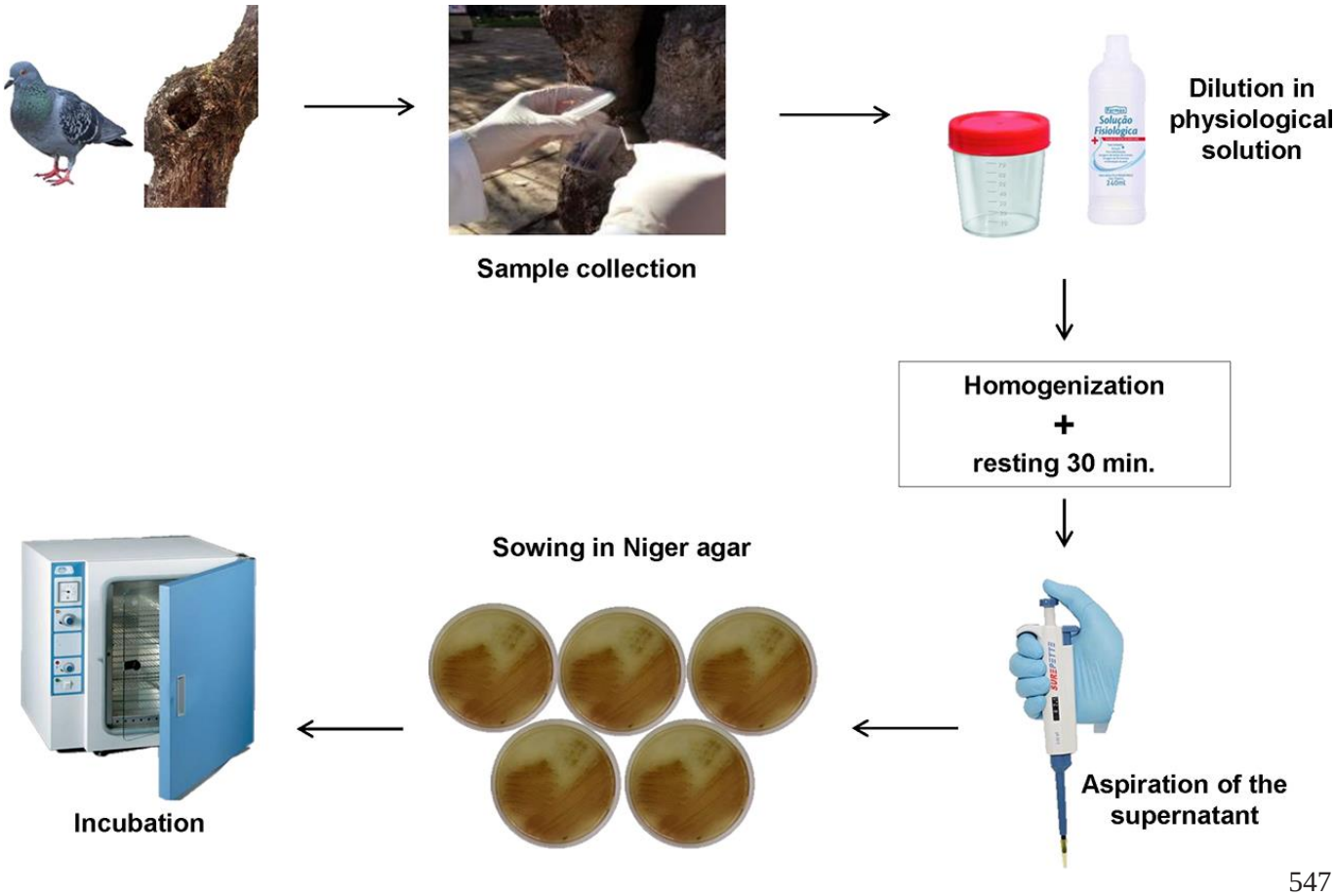
For this study, the total area was divided into two types of areas: academic areas and hospital areas. Academic areas were those composed of blocks of classrooms, amphitheaters, libraries, coffee shop, and other buildings located on campus. The hospital areas included the hospital and areas adjacent to it, such as corridors, ordinances, and parking lots.

*Samples Collection and Processing*

Samples of bird excreta were collected directly from the soil in 40 different spots, 19 of those were located in academic areas and 21 in hospital areas. Samples of tree hollows were collected from 23 trees, 10 of those were located in academic areas and 13 hospital areas. The samples were collected by picking up 2 to 5 grams of dried excreta or scraping tree hollows with the aid of previously sterilized metal spatulas.

**Figure 1.** Localization of the Clinical Hospital of Uberlandia in the city of Uberlandia, State of Minas Gerais, Brazil. Photo: Google Earth Pro.


**Figure 2.** Flowchart of the methodology used for sample collection and processing.



The organic matter was transferred to sterile flasks and sent, duly identified, to the laboratory for processing. One or two samples of each tree were obtained, depending on the presence of more than one hollow. It was possible to collect material from 41 different hollows belonging to 23 selected trees [23].

The samples were weighed and suspended in sterile physiological solution (NaCl 0.9%) containing 0.4 g/L chloramphenicol in the ratio of 1:10. They were ground with a glass rod and homogenized, and the suspension was allowed to stand for 30 minutes. After, 100 µL of the supernatant was aspirated using an automatic micropipette and deposited on the surface of a Petri dish containing Niger seed agar and chloramphenicol with a sterile Drigalski loop (Figure 2). Subsequently, they were incubated at 30 ºC for up to seven days with daily observation.

*Identification of isolates*

After growth of the colonies, we selected those that were yeast-like, had a mucoid consistency, were bright, and that acquired a beige to brown color. Then, they were transferred to tubes containing Sabouraud dextrose agar with chloramphenicol and later identified. The identification was conducted by the classical methodology, which included micromorphological study in cornmeal agar with tween 80, capsule formation, urease activity, and melanin production in Niger seed agar added to the assimilation tests of carbon and nitrogen sources [24]. Isolates that presented dubious identification or that were not identified by these means were submitted to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

MALDI-TOF MS

MALDI-TOF MS was performed according to the manufacturer's recommendations. Briefly, the colony grown in the culture medium was suspended in a tube containing 300 µL of distilled water, followed by addition of 900 µL of 99.5% alcohol and centrifugation at 13,000 rpm for 2 minutes. The supernatant was removed and then 20 µL of 70% formic acid was added. After vortex homogenization, 20 µL of acetonitrile was added, followed by centrifugation at 13,000 rpm for 2 minutes. Subsequently, measures and analyses were made in a mass spectrometer (MALDI-TOF, Bruker MALDI Biotyper 4.0). The criteria established for identification were: ≥ 2.0 for species and ≤ 1.7 for genus [25].

*Growth ability test at 37 ° C*

Growth ability at 37 °C was determined by inoculating yeast cells on Sabouraud dextrose agar. Results were reported after five days of incubation according to the presence or absence of growth. The test was performed in duplicate and incubated in temperatures of 30 °C and 37 °C.

## Results

A total of 81 samples were collected, 40 were from bird excreta and 41 from hollows, in the two areas (academic and hospital) included in the study. From the excreta of birds, 14 (35%) presented with growth of yeast of the genus *Cryptococcus*. Twenty isolates were identified including five (25%) *C. laurentii* (= *P. laurentii*); three (15%) *C. albidus* (= *N. albida*); three (15%) *C. liquefaciens* (= *N. liquefaciens*); two (10%) *C. friedmanii* (= *N. friedmanii*); one (5%) *C. curvatus* (currently *Cutaneotrichosporon curvatus*); and one each of the species (5%) *C. flavescens* (= *P. flavescens*),

**Figure 3.** Positivity of dry bird excreta samples and their isolates of *Cryptococcus* spp.

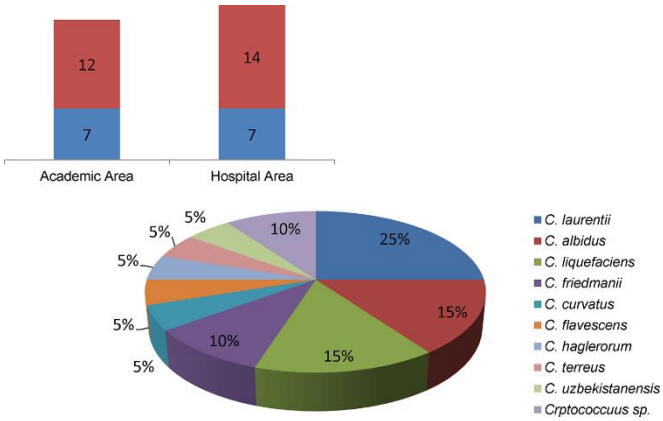
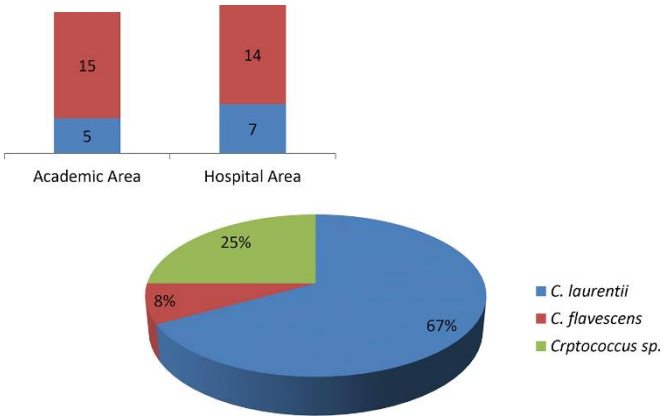



**Figure 4.** Positivity of tree samples and their isolates of *Cryptococcus* spp.



*C. haglerorum* (currently *Cutaneotrichosporon haglerorum*), *C. terreus* (currently *Solicoccozyma terreus*), and *C. uzbekistanensis* (currently *N. uzbekistanensis*). Additionally, two (10%) isolates were not identified (designated as *Cryptococcus* sp.). Simultaneous isolations of different species occurred in five excreta samples, with the following combinations: *C. laurentii* and *C. curvatus*; *C. laurentii* and *C. friedmanii*; *C. laurentii* and *C. albidus*; *C. uzbekistanensis* and *C. friedmanii*; and in another one, the following three isolates were found: *C. albidus*, *C. laurentii*, and *Cryptococcus* sp. (Figures 3 and 4).

From the 41 trees, there were 11 (27%) that presented with growth of yeast of the genus *Cryptococcus*, and the following 12 isolates were

**Table 1.** Distribution of species isolated by area and growth at 37ºC.

| Species (n) [currently name]* | Area A/H** n (%) | Sample | n | 37 °C n (%) |
|---|---|---|---|---|
| | H | T*** | 4 | 4 (57) |
| *C. laurentii* (13) | 7 (54) | E**** | 3 | 2 (28) |
| [*P.laurentii*] | A | T | 4 | 0 |
| | 6 (46) | E | 2 | 0 |
| | H | T | 0 | 0 |
| *C. albidus* (3) | 2 (67) | E | 2 | 0 |
| [*N. albida*] | A | T | 0 | 0 |
| | 1 (33) | E | 1 | 0 |
| | H | T | 0 | 0 |
| *C. liquefaciens* (3) | 1 (67) | E | 1 | 0 |
| [*N. liquefaciens*] | A | T | 0 | 0 |
| | 2 (33) | E | 2 | 0 |
| | H | T | 0 | 0 |
| *C. flavescens* (2) | 1 (50) | E | 1 | 1 (100) |
| [*P. flavescens*] | A | T | 1 | 0 |
| | 1 (50) | E | 0 | 0 |
| | H | T | 0 | 0 |
| *C. friedmannii* (2) | (0) | E | 0 | 0 |
| [*N. friedmannii*] | A | T | 0 | 0 |
| | 2 (100) | E | 2 | 1 (50) |
| | H | T | 0 | 0 |
| *C. curvatus* (1) | 1 (100) | E | 1 | 1 (100) |
| [*Cu. curvatus*] | A | T | 0 | 0 |
| | (0) | E | 0 | 0 |
| | H | T | 0 | 0 |
| *C. haglerorum* (1) | (0) | E | 0 | 0 |
| [*Cu. haglerorum*] | A | T | 0 | 0 |
| | 1 (100) | E | 1 | 0 |
| | H | T | 0 | 0 |
| *C. terreus* (1) | 1 (100) | E | 1 | 1 (100) |
| [*S. terreus*] | A | T | 0 | 0 |
| | (0) | E | 0 | 0 |
| | H | T | 0 | 0 |
| *C. uzbekistanensis* (1) | 0(0) | E | 0 | 0 |
| [*N. uzbekistanensis*] | A | T | 0 | 0 |
| | 1 (100) | E | 1 | 0 |
| | H | T | 2 | 1 (50) |
| *Cryptococcus* sp. (5) | 4 (80) | E | 2 | 0 |
| [not identified] | A | T | 1 | 0 |
| | 1 (20) | E | 0 | 0 |
| | H | T | (6) | 5(83) |
| Total Isolated | 17 (53) | E | (11) | 5(45) |
| (n = 32) | A | T | (6) | 0 |
| | 15 (47) | E | (9) | 1 (11) |

*[current name]: according to Liu et al. [18]; **A/H: academic area (A) and hospital area (H), respectively; ***T: trees; ****E: excreta.

identified: eight (67%) *C. laurentii*, three (25%) *Cryptococcus* sp., and one (8%) *C. flavescens*.

Regarding the test of growth capacity at 37 ºC, 11 (34%) isolates showed growth at this temperature (Table 1).

Twenty-three specimens of trees were analyzed, and 11 (48%) presented with growth of *Cryptococcus* spp. Of the tree species included in the study, 10 different species of trees were analyzed, and six (60%) presented with growth of *Cryptococcus* spp. (Table 2).

## Discussion

Several studies have shown the isolation of different *Cryptococcus* species of clinical importance from organic matter present in different environments. Species such as *C. neoformans sensu lato* and *C. gattii sensu lato* are generally the object of these researches, since they are the most frequent species that are etiological agents of cryptococcosis. However, when other species are sought and identified, it is observed in those studies that concomitant isolations of different species occur in the same analyzed sample analyzed, as they share a common ecological niche [26]. Thus, it is believed that the isolation of non-*neoformans* and non-*gattii Cryptococcus* spp. could be indicators of the presence of pathogenic species in the environment.

*Cryptococcus* infections occur worldwide and are acquired primarily by inhalation of fungal propagules present in ambient air. These fungi multiply in plants and bird excreta, especially from parrots, and propagules (dissected yeast or spores) spread with the wind, reaching animals and humans [27].

In this study, isolates of *C. neoformans sensu lato* and C. *gattii sensu lato* were not found. However, other species that have already been described as infectious agents of meningitis and pulmonary infections have been found [28,29,30]. These other species are part of a common niche shared with pathogenic species and may serve as markers of the presence of these pathogens. According to Khawcharoenporn *et al*. [31], *C. albidus* and *C. laurentii* together account for 80% of cases of cryptococcosis caused by non-*neoformans* and non-*gattii Cryptococcus* spp. and have traditionally been considered non-pathogenic to humans for many years. However, there have been more frequent reports in recent decades of opportunistic infections involving the skin, lung, bloodstream, and central nervous system whose agents have been *C. laurentii, C. albidus, C. curvatus, C. humicolus* (currently *Vanrija humicola*), and *C. uniguttulatus* (teleomorphic taxa: *Filobasidium uniguttulatum*) [18,32].

*C. albidus* have also been reported recently by Gyimesi *et al*. [33] in episodes of cutaneous infections with significant clinical manifestations. The expansion of this species can be justified by the increase in the number of individuals under immunocompromised conditions as well as by the population growth of birds in the urban environment, occasioned by the presence of solid waste, food, and humans that feed them, which favors its reproduction. Infections caused by these species are still rare, so these species have been the object of study for definitions of treatment and prophylaxis. Some studies have helped gain a better understanding of more effective antifungal therapies or new treatment alternatives [34,35,36].

*C. liquefaciens* is a species that has also been reported in episodes of infection [37,38], and it was the third most frequent species in our study. *C. liquefaciens*

**Table 2.** Relation between cryptococcal isolates and tree species.

| Trees | Number of specimens | Positives for *Cryptococcus* spp. n (%) | Isolates (n) | Isolates by area (n - %) |
|---|---|---|---|---|
| *Mangifera indica* | 3 | 1 (33) | *C. flavescens*[a] (1) | A* (1 - 100) |
| *Ligustrum lucidum* | 1 | 0 | 0 | 0 |
| *Bauhinia variegata* | 2 | 0 | 0 | 0 |
| *Ficus elastica* | 2 | 0 | 0 | 0 |
| *Caesalpinia peltophoroides* | 8 | 5 (63) | *C. laurentii*[b] (5)<br>*Cryptococcus* sp[c] (1) | A (2 - 33)<br>H** (4 - 67) |
| *Licania tomentosa* | 2 | 2 (100) | *C. laurentii*[b] (1)<br>*Cryptococcus* sp[c] (1) | A (1 - 50)<br>H (1 - 50) |
| *Tamarindus indica* | 1 | 1 (100) | *C. laurentii*[b] (1) | A (1 - 100) |
| *Araucaria heterophylla* | 1 | 1 (100) | *Cryptococcus* sp[c] (1) | H (1- 100) |
| *Tibouchina granulosa* | 2 | 1 (100) | *C. laurentii*[b] (1) | A (1 - 100) |
| *Terminalia catappa* | 1 | 0 | 0 | 0 |
| **Total** | **23** | **11 (48)** | **12** | **A (6 - 50)**<br>H (6 - 50) |

a: currently *Papiliotrema flavescens*; b: currently *Papiliotrema laurentii*; c: not identified; *A: academic area; **H: hospital area.

and *C. neoformans sensu lato* have close similarities in their structural composition, especially in the mucopolysaccharide capsule. Thus, the importance of the study of this species is justified, since they have virulence factors similar to those already considered for pathogenic species [39,40].

Other related studies carried out on bird excreta found in environments of big hospitals, such as those of Andrade-Silva *et al*. [6] and de Lima *et al*. [23], have also isolated fungi of the genus *Cryptococcus*. Therefore, it is possible to verify the exposure of numerous people, such as students, teachers, technicians, patients, and others, to infectious propagules of fungi.

In our study, isolates of *Cryptococcus* spp. occurred in samples collected from common places, such as hospital buildings and the central library of the university, where many people circulate daily. Figure 5 shows the geographic distribution of the isolates obtained from excreta and trees in the area of the study.

Lately, Spina-Tensini *et al*. [22] also reported a relationship between isolation sites and places frequented by people. Areas where there are large populations of pigeons and trees of the species *Araucaria angustifolia*, according to authors, would increase the likelihood of an individual developing cryptococcosis due to exposure to opportunistic species as well as the great load of infectious cells present in the environment.

Leftovers from food and garbage were found in entrances to the hospital. The presence of solid waste attracts urban birds, which facilitates their feeding and reproduction. Thus, these birds play an important role in the epidemiological chain, acting as vectors of these and other opportunistic pathogens to humans [41].

Understanding the epidemiology and pathogenesis of infections caused by non-*neoformans* and non-*gattii* *Cryptococcus* spp. contributes to the recognition of cryptococcal infections, helping with the early diagnosis and treatment of patients. It also leads to the search for new treatments and prevention schemes to reduce the incidence or avoid the aggravation of infection [32].

The low humidity of the air during the year, from May to September, allows the aerosolization of the infecting particles, which can reach high levels in the air, facilitating the inhalation of quantities that can lead to infection. Climatic and geographical factors are closely linked to the ecological niche of *Cryptococcus* as well as to its epidemiology. In their study, Cogliati *et*

**Figure 5.** Geographic distribution of *Cryptococcus* isolates obtained from bird droppings and tree debris in the study area. The region marked blue corresponds to the academic area, and the region marked red corresponds to the hospital area. The markings in yellow are isolated from bird excreta, and the markings in green are isolated from tree hollows.

*al*. [1] promoted a detailed forecast of the ecological niche for species of the genus *Cryptococcus*, which justifies the importance of establishing interdisciplinary actions with the purpose of understanding the risk that these yeast present to humans and to enumerate assertive and preventive actions.

## Conclusions

*Cryptococcus* spp. were found in the environments surveyed, which can infect humans. This reveals the diversity of their ecological niche, showing that these species survive and multiply in places closely related to humans and animals.

The handling and appropriate treatment of the environment, especially in the case of excreta, prevent the dissemination of great quantities of infectious particles. Individuals who may develop opportunistic infections (e.g., individuals with AIDS) should be informed about exposure to environments that are inhabited by infectious agents. This health education service is part of the responsibility of health service workers.

Thus, it emphasizes the importance of understanding the epidemiology of these occasional or emerging pathogens for public health, as a solution to broaden and improve the surveillance and control of cryptococcosis. Yeast of the genus *Cryptococcus* in both hospital and academic areas indicate the need to adopt preventive measures for promoting health and safety, for implementing health education strategies, and measures for controlling the bird population to prevent the transmission of diseases and spread of pathogens in the environment.

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