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Major Article

Environmental isolation, biochemical identification, and antifungal drug susceptibility of *Cryptococcus* species

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ABSTRACT

Introduction: The incidence of opportunistic fungal infections has increased in recent years and is considered an important public health problem. Among systemic and opportunistic mycoses, cryptococcosis is distinguished by its clinical importance due to the increased risk of infection in individuals infected by human immunodeficiency virus. **Methods:** To determine the occurrence of pathogenic *Cryptococcus* in pigeon excrement in the City of Araraquara, samples were collected from nine environments, including state and municipal schools, abandoned buildings, parks, and a hospital. The isolates were identified using classical tests, and susceptibility testing for the antifungal drugs (fluconazole, itraconazole, voriconazole, and amphotericin B) independently was also performed. After collection, the excrement samples were plated on Niger agar and incubated at room temperature. **Results:** A total of 87 bird dropping samples were collected, and 66.6% were positive for the genus *Cryptococcus*. The following species were identified: *Cryptococcus neoformans* (17.2%), *Cryptococcus gattii* (5.2%), *Cryptococcus ater* (3.5%), *Cryptococcus laurentti* (1.7%), and *Cryptococcus luteolus* (1.7%). A total of 70.7% of the isolates were not identified to the species level and are referred to as *Cryptococcus* spp. throughout the manuscript. **Conclusions:** Although none of the isolates demonstrated resistance to antifungal drugs, the identification of infested areas, the proper control of birds, and the disinfection of these environments are essential for the epidemiological control of cryptococcosis.

Keywords: *Cryptococcus* spp. Epidemiology. Antifungal Drugs. Environment.

INTRODUCTION

Of the more than 38 species of the genus *Cryptococcus*, two species are epidemiologically important. These species, *Cryptococcus neoformans* (serotypes A, D, and hybrid AD) and *Cryptococcus gattii* (serotypes B and C), differ genotypically, epidemiologically, and phenotypically, and they do not share ecological niches or geography[1].

Cryptococcosis is an opportunistic fungal disease that has increased in prevalence in recent years, accounting for 1 million infections and 700,000 deaths annually. Epidemiological data indicate that *C. neoformans* occurs most frequently in immunocompromised individuals, particularly those with acquired immunodeficiency syndrome (AIDS). In contrast, *C. gattii* is more common in healthy individuals[2].

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After establishing an infection in the lungs, these fungi can be eliminated by the immune system, thus causing an acute lung disease; they can remain in a latent state; or they can spread throughout the central nervous system (CNS) to cause meningitis or meningoencephalitis cryptococcosis[3,4].

Cryptococcus gattii has been isolated in tropical and subtropical regions. It was first isolated from *Eucalyptus camaldulensis* in Australia and was later isolated from plant material of other species of eucalyptus in different countries. In Brazil, *C. gattii* has been isolated from various plant species, such as *Ficus* and *Cassia grandis*, providing evidence that this species has a range of natural habitats[5].

Several studies have shown that *C. neoformans* remains viable in the dried excrement of birds, especially the excrement of species of the synanthropic pigeon, *Columba livia*[6-8]. These birds have found ideal conditions for survival in cities. Urban architecture, associated with an abundance of food, generates large areas of potential habitation and, consequently, large concentrations of excrement that can serve as potential sources of infection. The population of domestic pigeons has significantly increased in many parts of the world, including Brazil, and has become an environmental problem that directly influences public health[8,9].

Araraquara is located in the central region of São Paulo and has large green areas that serve as habitats for the wild dove,

which is commonly called *amargozinha* (*Zenaida auriculata*), and a large number of buildings and infrastructure promote infestation by domestic pigeons (*C. livia*). Additionally, a railway line passes through the center of the city, and the trains drop grain that serve as food for these species. Raso and colleagues[10] described an outbreak of cryptococcosis in birds in the State of São Paulo. They identified *C. gattii* in seven parrots, which was the first report of infection in these birds. These isolates were found to be resistant to fluconazole.

Drummond and colleagues[11] tested *Cryptococcus* strains isolated from clinical and environmental sources for resistance to azole fungicides that are commonly used in agronomy (i.e., epoxiconazole, difenoconazole, and cyproconazole) in comparison with the therapeutic antifungal agent fluconazole. The 50% minimum inhibitory concentration (MIC_{50}) values for the environmental isolates were greater than the MIC_{50} values for the clinical isolates. Thus, it is important that epidemiological studies evaluate the drug resistance of environmental isolates.

The goal of this study was to verify the presence of *Cryptococcus* spp. in various places in the City of Araraquara, SP, and to assess the susceptibility of these isolates to the antifungal drugs that are used in the treatment of cryptococcosis.

METHODS

Sample collection

We collected 87 samples of bird droppings from different locations in the City of Araraquara, SP, from 22/07/2009 to 03/12/2011. Approximately 10g of each sample of excrement was collected. The specimens were placed in sterile tube collectors, standardized, and sent to the Clinical Mycology Laboratory of the *Universidade Estadual Paulista* (UNESP), Araraquara, Faculty Pharmaceutical Sciences, Campus of Araraquara, State of São Paulo, Brazil.

Isolation and identification of the yeast

To isolate *Cryptococcus* spp., 1g of the biological material was weighed and placed into Falcon tubes containing 10ml of saline solution (0.85% NaCl) and 0.05g/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, the 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, and growth on canavanine-glycine-bromothymol blue medium. For the biochemical tests, the auxanogram 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).

Susceptibility testing

The MICs and minimum fungicidal concentrations (MFCs) were determined for the antifungal drugs amphotericin B, itraconazole (Jansen Pharmaceuticals, Beerse, Belgium), voriconazole, and fluconazole (Pfizer International, New York, NY) were tested against all of the isolates. The MIC test was conducted according to the Clinical and Laboratory Standards Institute (CLSI) document M27A2 (2002) with several modifications. Antifungal susceptibility testing was performed according to the microdilution method recommended by the CLSI. The final concentrations of the drugs ranged from 0.06 to 64μg/ml^{-1} for fluconazole and from 0.015 to 16μg/ml^{-1} for itraconazole, voriconazole, and amphotericin B. *Candida albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were included as controls to assess the reproducibility of the results.

RESULTS

Environmental isolates

A total of 87 environmental samples collected, and 66.6% (58) were positive for the genus *Cryptococcus* when analyzed using direct microscopy with Chinese ink, the urease test, and the phenol oxidase production test. Of the collection sites with positive samples, 10.3% (6) were schools; 10.3% (6) were parks, squares, or streets; 24.2% (14) were abandoned shacks; and 55.2% (32) were hospitals or in close proximity to hospitals.

Of the 58 strains tested, 3.5% (2) were identified as *C. ater*, 1.7% (1) as *C. laurentii*, 1.7% (1) as *C. luteolus*, 5.2% (3) as *C. gattii*, 17.2% (10) as *C. neoformans,* and 70.7% (41) as *Cryptococcus* spp; these strains were identified using the auxanogram test (**Table 1**). In terms of collection sites, 6.9% (6) of the positive samples were collected from schools (sites A and B); 6.9% (6) from parks, squares, and streets (sites C and D); 20% (14) from abandoned shacks (site E); and 36.8% (32) from hospitals or in close proximity to hospitals (sites F and G). The auxanogram test was not able to determine the species of all of the environmental isolates. Of the 58 strains tested, 3.5% (2) were identified as *C. ater*, 1.7% (1) as *C. laurentii*, 1.7% (1) as *C. luteolus*, 5.2% (3) as *C. gattii*, and 17.2% (10) as *C. neoformans*, and 70.7% were classified as *Cryptococcus* spp. **(Table 1)**. The relative percentages of positive isolates at each of the collection sites were also analyzed. In public schools, 20% of the samples collected at site A and 66.7% of the samples from site B were positive for *Cryptococcus*. In parks, squares, and streets, 44.4% of the samples from site C and 50% of the samples from site D were positive. Of the samples collected at abandoned shacks (site E), 70% were positive. We observed that 85.7% of the samples from site F and 66.7% of the samples from site G **(Table 1)** collected in the proximity of hospitals were positive.

Susceptibility testing

The environmental isolates identified as *C. neoformans* showed amphotericin B MIC and MFC values ranging from 0.3125 to 0.125μg/mL. The MIC values of these isolates ranged from 2.0 to 8.0μg/mL for azoles, and the MFC values ranged

TABLE 1 - Collection sites, number of samples collected, geographic coordinates, environmental niches of the city and region, and absolute and relative isolations of *Cryptococcus* in Araraquara, State of São Paulo, Brazil.

Collection sites	Number of samples collected/positive samples	Region	Environmental niche	Collected sample/positive	Absolute isolation (%)/relative isolation (%)
Public School (site A)	10/02	East	*C. livia* droppings	10/02	2.3/20.0
Public School (site B)	06/04	Southwest	*C. livia* droppings	06/04	4.6/66.7
Central Region Plaza (site C)	09/04	Central	*Z. auriculata* droppings	09/04	4.6/44.4
Central Region Park-Playground (site D)	04/02	Central	*Z. auriculata* droppings	04/02	2.3/50.0
Abandoned shed (site E)	20/14	Central	*C. livia/Platyrrhinus lineatus* droppings and excrement	20/14	16.1/70.0
Psychiatric Hospital (site F)	35/30	East	*C. livia* droppings and excrement	35/30	34.5/85.7
Hospital (site G)	03/02	Central	*C. livia* droppings	03/02	2.3/66.7
Samples Total				87/58*	100.00/-

C. livia: Columba livia; Z. auriculata: Zenaida auriculata. *Of 58 positive samples, 41 samples were not identified through the auxanogram test. Therefore, these isolates were listed as *Cryptococcus* spp.

from 4.0 to 32.0μg/mL. For itraconazole, the MIC and MFC values were 0.625μg/mL, and the voriconazole MIC and MFC values ranged from 0.0625 to 4.0μg/mL. The *C. gattii* isolates had amphotericin B and itraconazole MIC and MFC values of 0.0625μg/mL. Fluconazole exhibited fungistatic activity against these isolates with MIC and MFC values of 4.0μg/mL and 16.0μg/mL. In contrast, voriconazole was more active and had MIC and MFC values of 0.0625 and 4.0μg/mL, respectively. The isolate identified as *C. luteolus* had MIC and MFC values for amphotericin B and itraconazole of 0.0625μg/mL. The fluconazole MIC and MFC values for this isolate were 4.0μg/mL, and the voriconazole MIC and MFC values were 1.0μg/mL and 2.0μg/mL, respectively. The isolates identified as *C. laurentii* and *C. ater* had amphotericin B and itraconazole MIC and MFC values of 0.0625μg/mL. The MICs of fluconazole for *C. laurentii* and *C. ater* were 4.0μg/mL for both species, and the MFCs of fluconazole were 16.0μg/mL for *C. ater* and 8.0μg/mL for *C. laurentii*. These isolates had voriconazole MIC values of 1.0 μg/mL, and the voriconazole MFC value was 1.0μg/mL for *C. laurentii* and 2.0μg/mL for *C. ater* **(Table 2)**.

TABLE 2 - MIC and MFC values of environmental isolates of *Cryptococcus* spp. to the antifungal drugs amphotericin B, fluconazole, itraconazole and, voriconazole.

Number of isolates	Range			
	amphotericin B MIC/MFC (μg/mL)	fluconazole MIC/MFC (μg/mL)	itraconazole MIC/MFC (μg/mL)	voriconazole MIC/MFC (μg/mL)
C. neoformans (52 isolates)	0.03125 - 0.125/0.03125 - 0.125	2.0 - 8.0/4.0 - 32.0	0.0625/0.0625	0.0625 - 4.0/0.0625 - 4.0
C. gattii (3 isolates)	0.0625/0.0625	4.0/16.0	0.0625/0.0625	0.0625 - 1.0/0.5 - 2.0
C. luteolus (1 isolate)	0.0625/0.0625	4.0/4.0	0.0625/0.0625	1.0/2.0
C. laurentii (1 isolate)	0.0625/0.0625	4.0/16.0	0.0625/0.0625	1.0/1.0
C. ater (1 isolate)	0.0625/0.0625	4.0/8.0	0.0625/0.0625	1.0/2.0
ATCC 90012	0.5/0.5	4.0/8.0	0.0625/0.0625	0.5/1.0

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; *C.: Cryptococcus.*

DISCUSSION

Cryptococcosis is a global public health concern. A study conducted in 1976 by Swinne-Desgain[12] indicated that birds play an important role in the epidemiology and spread of the disease because many pigeons carry yeast in craw, remaining viable for up to 86 days. In 2002, Filiu and colleagues[13] detected 46,000 viable propagules of *C. neoformans* var. *neoformans* per gram of dry pigeon excrement, indicating the existence of microfocal environmental sources.

Rosario and coworkers[14] analyzed samples from vent pigeons to identify the presence of *C. neoformans*; this yeast was identified in 1.8% of the total samples. In a later study, the same author confirmed the relationship between *C. neoformans* and *C. livia* pigeons and demonstrated the importance of birds as a reservoir of pathogenic forms of *C. neoformans*.

Cryptococcus neoformans is cosmopolitan and may occur in many organic substrates associated with bird habitats, including dry droppings, which are rich in urea and creatinine. Thus, areas infested with pigeons become an important ecological niche because the dispersion and adaptation the pigeon species *C. livia* and *Z. auriculata* are notorious in urban centers. Another factor that may contribute to the maintenance and dissipation of *C. neoformans* in urban centers is the presence of birds in residential and commercial areas. Our results showed that of the 87 samples collected, 66.7% were positive for *Cryptococcus* spp. This result is similar to those of several studies in Brazil[7,15,16]. It was not possible to determine the species of all environmental isolates using the auxanogram test. Of the 58 strains tested, 3.5% (2) were identified as *C. ater*, 1.7% (1) as *C. laurentii*, 1.7% (1) as *C. luteolus*, 5.2% (3) as *C. gattii*, and 17.2% (10) as *C. neoformans,* and 70.7% were classified as *Cryptococcus* spp. Our data agree with a 2010 report from Costa et al.[17] that described the attempt to isolate *Cryptococcus* spp. from 47 urban pigeon dropping samples and from 322 samples collected from the cloacae of the birds; *C. neoformans* was isolated only from the pigeon dropping samples collected in the environment. The connection between the distribution of *C. neoformans* and pigeons has been previously described. However, whether pigeons are infected or serve as carriers for *C. neoformans* is still debatable[18,19]. In 2008, Lugarini et al.[20] published a study in which serum samples from *Psittacine* and *Columbiformes* species were examined for the presence of *C. neoformans* polysaccharide antigens. Of the 53 pigeon serum samples collected, only one (1.8%) sample (from *C. livia*) was positive. Baroni et al.[21] evaluated the presence of *C. neoformans* in 10 churches in Rio de Janeiro. They collected pigeon dropping and air samples from church towers and the surrounding areas for a year. The researchers observed that the fungus was present in all of the churches and in 37.8% of the 219 samples of pigeon droppings. Soares et al.[22] examined 79 samples of pigeon excreta in the City of Santos, São Paulo, and found that 11 (13.9%) samples were positive for *C. neoformans*. Of these 11 samples, four were from church towers, and seven were obtained elsewhere. The preferred environmental niche identified for the presence of *C. neoformans* in the present study involved enclosed, poorly ventilated spaces that were protected from sunlight, such as abandoned buildings. Open areas that are ventilated and unprotected from the sun did not show the presence of *C. neoformans*; similar results have been described for environmental studies of *Cryptococcus* in Votuporanga, SP; the occurrence of *C. neoformans* in pigeon droppings in the City of Pelotas, RS; and the isolation and identification of *C. neoformans* in pigeon excreta from public and residential places in Great Plains and Cuiabá, MT[7].

The isolation and identification of the pathogenic species *C. laurentii* and *C. luteolus* demonstrates the importance of biodiversity and the identification of the environmental niches of pathogenic species because these species can increase the risk of infection of immunocompromised patients.

In 2002, Averbuch et al.[23] isolated and identified *C. laurentii* from the blood of a patient diagnosed with a ganglioneuroblastoma. This study is very important because it demonstrated the presence of this specie in a patient with cancer and determined that the isolate was resistant to fluconazole[23]. In 2006, Shankar et al.[24] reported a case of pulmonary cryptococcosis caused by *C. laurentii* in a diabetic AIDS patient who was on antituberculosis and antiretroviral treatments. Another case of fungemia caused by *C. laurentii* was reported in a young man with membranoproliferative glomerulonephritis who was on an aggressive immunosuppressive therapy[25]. Research conducted by Leite et al.[26] published in 2012[26] described the presence of *Cryptococcus* spp. on dust found on books in three libraries in the City of Cuiabá in Mato Grosso, Brazil. Of the 84 samples collected from the book dust, 18 (21.4%) were positive for *Cryptococcus* spp. The most frequently isolated species was *C. gattii* (15; 36.6%), followed by *C. terreus* (12; 29.3%), *C. luteolus* (4; 9.8%), *C. neoformans* and *C. uniguttulatus* (3; 7.3%), and *C. albidus* and *C. humiculus* (2; 4.6%).

A study in Pelotas, State of Rio Grande do Sul, isolated samples from sources with large amounts of droppings, such as church spires, rice mills, warehouses, parks, historic buildings, and outdoor locations. However, only one sample tested was positive for *C. neoformans*[7].

In 2011, Pfaller et al.[27] determined MIC values as cutoffs of 0.25, 0.12, and 8.0mg/L for *Cryptococcus* spp. for the drugs posaconazole, itraconazole, and fluconazole, respectively. We evaluated the susceptibility of 58 environmental isolates of the genus *Cryptococcus* to the frontline antifungal drugs fluconazole, itraconazole, voriconazole, and amphotericin B to detect any possible resistance. We observed that all of the isolates were susceptible to the antifungal agents tested. Drummond et al.[11] examined the susceptibility of several strains of *Cryptococcus*, including environmental isolates, to azole compounds and found that the environmental isolates had increased resistance to these compounds. Thus, it is assumed that the decreased susceptibility of most environmental isolates to azoles is due to the possible environmental contamination by these compounds because the locations where the samples were collected included squares, parks, schools, and hospitals that used gardening services. Kobayashi et al.[15] tested 36 environmental isolates of *C. neoformans* in the City of Goiânia,

Goiás State for resistance to amphotericin B, fluconazole, and itraconazole and did not identify any resistant isolates. The same results were reported in a study by Souza et al.[28] that evaluated 70 clinical isolates and 40 environmental isolates of *C. neoformans* GO; no environmental isolates showed resistance to amphotericin B, itraconazole, fluconazole, or ravuconazole. These susceptibility data corroborate the data in our study; despite finding a range of MIC values, all of the isolates we tested were classified as susceptible to the antifungal agents tested. Trpković and collaborators[29] evaluated the antifungal susceptibilities of 31 *C. neoformans* clinical isolates collected in Serbia over a 10-year period. The strains were isolated from the cerebrospinal fluid and blood of patients with AIDS or lymphoma and from a kidney transplant recipient. The MICs of amphotericin B, 5-fluorocytosine, fluconazole, and itraconazole were determined. The isolates were highly susceptible to amphotericin B (100% susceptibility at MIC $< 0.5\mu g/mL$) and 5-fluorocytosine (87.1% susceptibility at MIC $\leq 4\mu g/mL$). Fluconazole exhibited the lowest activity *in vitro* (48.4% susceptibility at MIC $\leq 8\mu g/mL$) and had a significant resistance rate. The activity of itraconazole was also decreased (48.4% susceptibility at MIC $\leq 0.25\mu g/mL$). According to the authors, the low rate of susceptibility to fluconazole stresses the need for active *C. neoformans* antifungal surveillance and for corresponding data from different geographic regions.

A total of 176 (95 clinical and 81 environmental) *C. neoformans* isolates and eight clinical *C. gattii* isolates were evaluated by Andrade-Silva et al. in 2013[30] to determine the MICs. A total of 10.5% of *the C. neoformans* clinical isolates were resistant to amphotericin B, and 6.2% of the environmental isolates were resistant to fluconazole. All of the *C. gattii* isolates showed high susceptibility to most of the antifungals evaluated. The clinical isolates were less susceptible than the environmental isolates to amphotericin B and itraconazole, and the environmental isolates were less susceptible than the clinical isolates to fluconazole, voriconazole, and ketoconazole.

In the face of the information obtained in this study, we suggest that further epidemiological studies of *Cryptococcus* species are essential to identify the foci of this fungus, and that preventive measure, such as the control of carriers, especially birds, should be undertaken.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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