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Isolation of Cryptococcus spp. from several environmental niches in São Luís, MA

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ABSTRACT

Cryptococcosis is an invasive mycosis triggered by a complex of fungal pathogens present in various environmental niches. *Cryptococcus neoformans, C. gattii,* and emerging pathogens such as *C. laurentii* and *C. albidus* are found in aged excreta of *Columba livia* (pigeon), its natural disseminator. As the pigeon population has increased in São Luís, the objective of this research was to demonstrate the presence of Cryptococcus spp. in the excreta of *C. livia* in public environments. Twenty-three samples were collected at 14 sites, dispensed into conical tubes, homogenized with saline and chloramphenicol, and allowed to rest until processing. Twenty-four hours after collection, aliquots were distributed in a fungal culture medium and incubated. The macromorphological examination revealed levaduriform, mucoid, bright, isolated colonies compatible with Cryptococcus spp. In the micromorphological examination, 11 of the 23 samples (42.85%) showed the presence of cells with a thick, refringent capsule and mucopolysaccharide around the blastoconidia, typical of Cryptococcus spp. fungi. The other samples (57.14%) were negative for the fungus. The environmental isolation of this fungus in public areas is relevant to public health since the growing pigeon population in São Luís increases the risk of exposure and infection by dispersion of infectious propagules in the environment.

Keywords: Mycoses; Pigeons; Cryptococcus spp

1 INTRODUCTION

Cryptococcosis, also known as torulosis, European blastomycosis, Busse-Buschke disease, or pigeon disease, is an invasive fungal infection that affects both humans and animals. The disease is mainly triggered by two distinct free-living encapsulated yeasts belonging to the division of basidiomycetes, namely, *Cryptococcus*

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neoformans and *Cryptococcus gattii*. However, other species have been described as emerging pathogens and disease triggers. These species have probably evolved as accidental pathogens given a selective environmental pressure (MAY *et al*., 2016). The *C. neoformans/gattii* complex comprises two species subdivided and classified into different genotypes. These species were in turn, separated into serotypes based on the various capsular immunological properties. *C. neoformans* was classified in two serotypes, namely, serotype A (also called *grubii* variation) and serotype D (*C. neoformans* var. *neoformans*) (MIRANDA *et al*., 2014). Hybrid microorganisms called AD are also present in the *C. neoformans* species (FRANZOT *et al*., 1999), whereas *C. gattii* includes serotypes B and C (BOEKHOUT *et al*., 2001; KWON-CHUNG; VARMA, 2006). Molecular technique studies have confronted and questioned the use of only two species in the *C. neoformans/gattii* complex. Hagen *et al*. (2015) used phylogenetic analysis and genotyping and proposed recognizing the current *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* as two different species and *C. gattii* as five separate species. *Cryptococcus* is present in numerous environmental niches, such as wooded sites), soil, squares, outdoor and indoor areas of hospitals, condominia, vegetables, household dust, and aged bird excreta, more precisely pigeon (*Columba livia*) excrements. Pigeons represent a potential zoonotic risk to public health and in massive agglomerations of these animals, the large concentration of local dirt by both excreta and food scraps poses a risk to human health (MIRANDA *et al*., 2014).

Pigeons are concentrated in places where ample food supply is provided by local people, contributing to bird proliferation in urban areas (SCHULLER, 2005). Excreta are rich in urea and creatinine, which favors the development of the fungus. Besides, *Columba* spp. are natural disseminators of yeasts, because these are present in their feathers, feet, and beaks (LIN, 2009). The environment has been considered as the only potential source of infection when individuals inhale the infectious propagules or have traumatic open lesions (CHAYAKULKEEREE; PERFECT, 2006). Aerosolization of the propagules may favor inhalation by the host causing systemic hematogenous infections, extensively reaching organs such as the liver, myocardium, lungs, prostate, urinary tract, eyes, skin, joints, bones, and the central nervous system (CNS), being commonly lethal in both immunosuppressed and immunocompetent individuals. *Cryptococcus* spp. has an affinity for the CNS, triggering meningoencephalitis and contributing to the morbidity/lethality of the disease. Cryptococcal meningitis is considered the third most frequent neurological complication in patients with acquired immunodeficiency syndrome (AIDS) at an advanced stage of the disease (DELL VALE; PINA-OVIEDO, 2006).

Cryptococcosis is considered an opportunistic cosmopolitan infection coexisting with other pathologies. The disease is mainly caused by *C. neoformans* in immunocompromised patients or by *C. gattii* through endemic infection in immunocompetent individuals. In addition, other infections and clinical manifestations with distinct etiology are caused by *C. neoformans* serotype A in immunocompetent individuals or by *C. gattii* in patients with AIDS (KWON-CHUNG; BENNETT, 1992; LAZÉRA *et al*., 2009). The geographical distribution of these fungi was previously restricted to the tropical and subtropical regions. However, the presence of *C. gattii* has been reported in temperate regions in an epidemic caused by the pathogen at Vancouver Island, Canada (CHAKRABARTI, 1997; MACDOUGALL, 2011; KIDD, 2004). *C. gattii* is endemic in North and Northeastern Brazil with lethality between 35% and 40% of confirmed cases. Clinical and epidemiological studies conducted in the country revealed the clinical importance of *C. gattii* neurocryptococcosis in young adults of both sexes and children. Cases of infection caused by the VGII genotype, adapted to urban environments, and with an endemic–epidemic behavior were reported in Rio de Janeiro (LAZÉRA, 1995; LACAZ, 2002; TRILLES, 2008; LEMOS, 2005). The pathology is not of compulsory notification, making it difficult to determine the extent of the disease in Brazil.

Cryptococcus spp. infections have significant worldwide relevance and indices given the increase in immunosuppressed patients at global levels. Recently, Nature Microbiology published an editorial (2017) that highlighted the need of not neglecting fungi. Through this study, the authors proposed a reflection on fungi and how these microorganisms have been neglected in spite of well-established studies showing their medical relevance.

Maranhão, in the Northeast of Brazil, has an endemic profile of infectious and systemic fungal diseases. In the capital city, the unbridled proliferation of pigeons has frequently caught the attention of the media because of the constant complaints of the community. However, a method for bird control is not yet implemented in São Luís, and so pigeons proliferate posing a public health problem. Another aggravating factor is that the emergence of the etiological agent is strongly related to environmental climate alternations. São Luís is a region with a hot, humid, and tropical weather that favors vegetation; thus, these conditions promote the emergence of the pathogen in the region. There is also a new geographic distribution of these fungi including the presence of *C. gattii* in temperate regions, causing epidemic outbreaks in areas not previously affected by the pathogen. Considering the clinical importance of the pathogen, the objective of this study was to demonstrate the presence of *Cryptococcus* spp. in *C. livia* excreta from different public environments in São Luís, Brazil.

2 MATERIALS AND METHODS

2.1 Geographic Localization

According to data from the Brazilian Institute of Geography and Statistics, IBGE (2010) the great São Luís is the main city of the Metropolitan Region and has 1,014,837 inhabitants with a demographic density of 1,215.69 inhabitants/km^2). The city occupies an area of 834,785 km^2 and is located in Northeastern Brazil, South of Ecuador, and 24 meters above sea level. Its climate is tropical semi-humid, strongly influenced by the sea and the Intertropical Convergence Zone. It is the only Brazilian city founded by the French, being one of the three Brazilian capitals located on islands (the others are Florianópolis and Vitória). The most populous city is Maranhão, the 15th most populous municipality in Brazil, and the fourth from the Northeastern Region. The city has a large number of coconut trees and coastal vegetation, with areas of the Amazon Rainforest that still resist urbanization. There are also small rivers that originate in the city, with the Bacanga River being the most important for the economy.

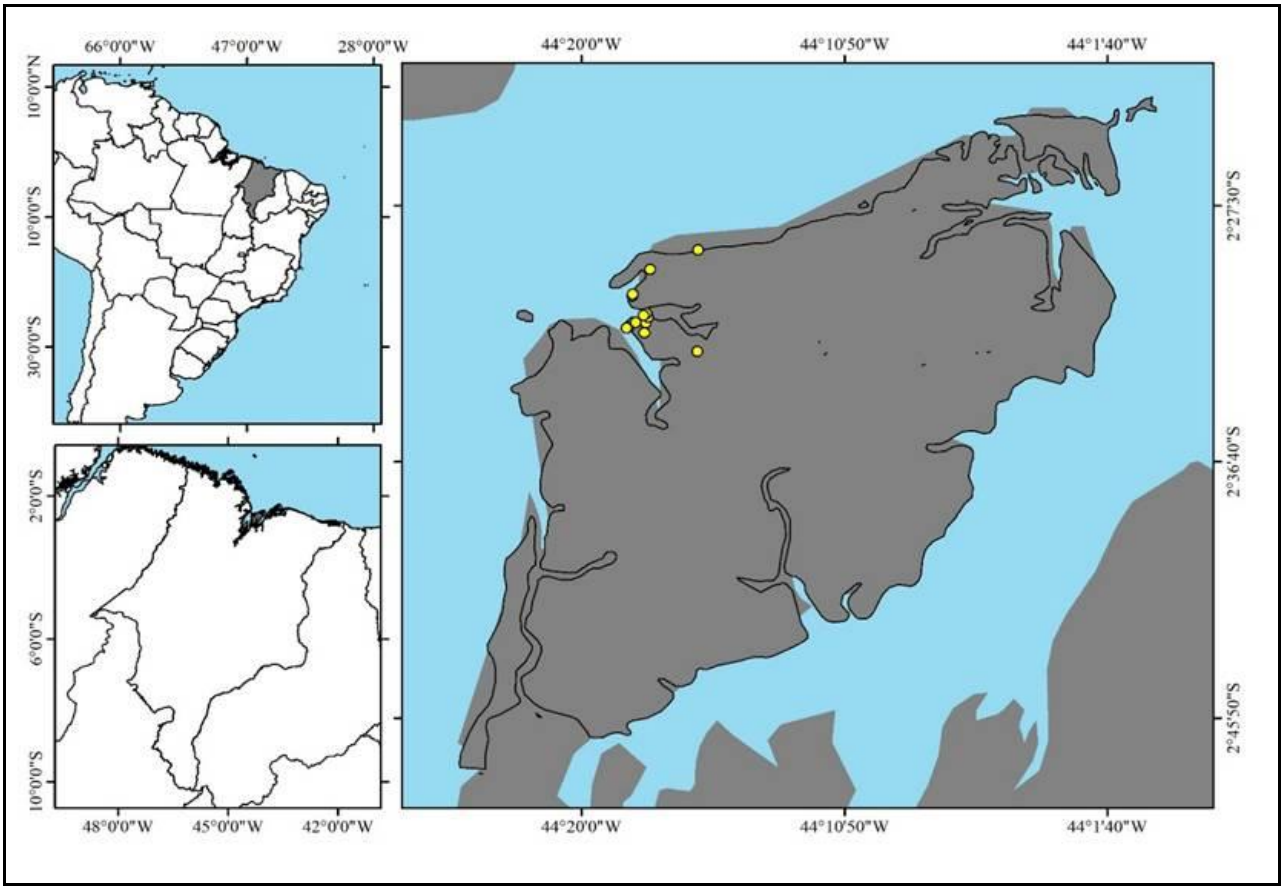
2.2 Season and Collection Sites

Sample collection took place from September 2017 to March 2019, comprising both dry and rainy seasons. The regions chosen for excreta collection included central areas of the capital and surroundings, as well as squares, hospitals, clinics, commercial buildings, fairs, waterfront, and other places where pigeons and large movement of people, including food hawkers, were present. The sites were chosen based on observational evidence from previous visits and through the city communication systems that always convey these regions as being the most populated by pigeons.

Quantum GIS 3.6.2® software was used for data processing in order to georeference the collection sites of the 23 dry and semi-solid samples of pigeon excreta obtained at the following points: Clinic in the city center (CC), Santa Casa de Misericórdia Hospital (HSCM), Getúlio Vargas Hospital (HGV), Presidente Dutra Hospital (HPD), Odorico Mendes Square (POM), Maria Aragão Square (PMA), Nauro Machado Square (PNM), San Francisco Square (PSF), João Lisboa Square (PJL), Ribeirão Fountain Square (PFR), São Francisco Commercial Building (PCSF), Praia Grande (PG), Litorânea Avenue (AVL), and Jansen Lagoon (LJ) (Figure 1).

The average number of pigeons and excreta were recorded. The amount of excreta was semi-quantified using the following scores: 00, no excreta; 01, few excreta; 03, median amount; 05, large amount with accumulation in places. The number of pigeons was assessed using the following scores: 00, absence of pigeons; 01, from 10 to 30 pigeons; 02, from 31 to 50 pigeons; 03, more than 50 pigeons (Table 1). These two scores were analyzed with Biostat 5.3® using the Spearman correlation coefficient for nonparametric variables. Spearman correlation evaluates the relationship between continuous or ordinal variables. These variables tend to change together but not necessarily constantly. Spearman correlation coefficient is based on the classified values assigned to the variables rather than to the raw data.

Figure 1 - Location of the study area: São Luís, Maranhão State, Brazil. The yellow balls indicate the collection points. CC, Hospital SCM, Hospital GV, Hospital PD, Praça OM, Praça MA, Praça NM, Praça SF, Praça JL, Praça FR, Prédio CSF, Praia Grande - PG, Av. Litorânea - AVL e Lagoa da Jansen - LJ



2.3 Sample Collection and Processing

Twenty-three dry and semi-solid pigeon excreta samples were collected from 14 different sites. Samples were evaluated at all selected sites, taking into account parameters such as solid and semi-solid excreta on public surfaces, presence of pigeons close to excreta and absence of other birds, presence of chicks or nests, and approximate amount for processing. The excreta collections were made on the ground surface and on diverse objects, the most prevalent being benches of concrete and wooden squares, cement sidewalks, statues of squares, air conditioning boxes, walls and sides of bars and windows, walls, roofs, and others.

Sterile spatulas and collectors were used to remove samples from the collecting sites. Samples were then packed, identified by location, and transported to the laboratory for processing. Isolation of fungi was carried out within 24 h after collection. Samples were processed in a safety cabinet and macerated with the aid of a sterile grail and pistil, until a fine and homogeneous consistency was obtained. Feather and other residues were removed from the excrement and approximately 1 g of the feces placed into conical tubes. Fifty milliliters of 0.9% sterile saline solution with 0.4 g/L of chloramphenicol (to inhibit bacterial growth) were added to each tube. The material was further stirred for 3 min and allowed to stand for 30 min. Afterward, 10 µL, 100 µL, 200 µL, and 500 µL of the supernatant were mixed with glass beads and spread onto Niger and Sabouraud Agar plates containing 0.5 mg chloramphenicol. Plates were incubated in a greenhouse at 30 ºC for up to 3 days and checked daily for colony appearance. Isolated colonies were streaked onto Sabouraud Agar tubes to maintain the sample. Subsequently, samples were subjected to a micromorphological test by negative staining. This technique consists of a direct microscopic observation of a smear made with a colony loopful and a drop of Chinese ink onto a glass slide after mounting a coverslip.

2.4 Statistical Analysis

We performed a Spearman rank correlation analysis, for amount of excreta and the number of pigeons in the research site. A p value of < 0.05 was considered significant.

3 RESULTS AND DISCUSSION

The macromorphological examination of isolated colonies showed bright and mucoid yeast colonies compatible with *Cryptococcus* spp. A direct microscopic examination of isolates was carried out for visualization of the micromorphological structures. *Cryptococcus* structures do not stain with Chinese ink because of the thick mucopolysaccharide capsule of the fungus. The pigment particles do not enter the capsule resulting in bright, round, and oval structures on a dark background. The micromorphology showed the presence of cells with a refringent, thick mucopolysaccharide capsule surrounding round blastoconidia, typical of *Cryptococcus* spp. The test was positive for 11 out of the 23 processed samples from the 14 collection sites (42.85%) and negative for the remaining 12 (57.14%) (Table 1).

Table 1 - Identification of collection sites separated by localities, quantitative assignment of pigeon and excreta scores, and colony evaluation and microscopy results

Place	Excreta scores	Quantitative assignment of pigeon	Results
–CC Clinic	03	01	suggestive for Cryptococcus spp.
	03	01	suggestive for Cryptococcus spp.
	03	02	suggestive for Cryptococcus spp.
Hospital SCM	05	03	suggestive for Cryptococcus spp.
	00	01	No samples to process
	05	03	suggestive for Cryptococcus spp.
Hospital GV	01	02	negative for Cryptococcus spp.
	00	01	No samples to process
	01	03	negative for Cryptococcus spp.
Hospital PD	03	02	negative for Cryptococcus spp.
	01	02	negative for Cryptococcus spp.
	01	01	negative for Cryptococcus spp.
OM Square	01	02	suggestive for Cryptococcus spp.
	00	00	No samples to process
	00	00	No samples to process
MA Square	01	02	negative for Cryptococcus spp.
	03	03	suggestive for Cryptococcus spp
	05	03	suggestive for Cryptococcus spp.
NM Square	01	01	negative for Cryptococcus spp.
	00	00	No samples to process
	00	01	No samples to process
SF Square	00	02	No samples to process
	00	02	No samples to process
	03	03	suggestive for Cryptococcus spp.
JL Square	00	01	No samples to process
	03	01	negative for Cryptococcus spp.
	01	02	negative for Cryptococcus spp.

Continues...

Table 1 - Identification of collection sites separated by localities, quantitative assignment of pigeon and excreta scores, and colony evaluation and microscopy results

Conclusion

Place	Excreta scores	Quantitative assignment of pigeon	Results
FR Square	00	01	No samples to process
	00	00	No samples to process
	01	01	negative for Cryptococcus spp.
Praia Grande - PG	00	01	No samples to process
	00	01	No samples to process
Litorânea Avenue - AVL	01	02	negative for Cryptococcus spp.
	01	01	negative for Cryptococcus spp.
Lagoa da Jansen -LJ	00	01	No samples to process
	03	05	suggestive for Cryptococcus spp.

Large amounts of food, such as corn and bread, were observed in two squares of the research sites. The food is supplied by local people who feed the pigeons resulting in an increase in the bird population and an increase in the risks of exposure. In some places, neither pigeons nor excreta were found during the rainy season, suggesting that climate alternations may have influenced the non-emergence of the vector and the concomitant presence of the pathogen in the region under study. The same sites were populated by pigeons during drought.

The statistical test showed a significant ($p < 0.0001$) and positive Spearman correlation ($r = 0.6918$) between the amount of excreta and the number of pigeons in the research site. However, at some locations a 01 score for pigeons together with a 00 score for excreta were recorded during a period of higher precipitation, suggesting that excreta may have been removed by the rain or that the absence of anthropogenic activities such as feeding may have interfered with the subsequent accumulation of excreta at the site. In addition to the fungus objective of this study, two more fungi were found: *Candida* spp. and *Rhodotorula* spp. These microorganisms can also be isolated from some environmental niches where *Cryptococcus* spp. can be lethal to human beings depending on the immune condition of the host.

In São Luís, the unbridled proliferation of pigeons has been also observed at the seafront, which favored the interest in studying the phenomenon in this area as well. Currently, as the corresponding government agencies do not have a method to control bird reproduction, pigeons proliferate to the point of being considered a public health problem. There are numerous environmental niches where the *Cryptococcus* spp. fungi proliferate, and these are strongly linked to the various organic substrates that the fungus can use, including bird excreta. Staib (1964) showed that isolation of *Cryptococcus* spp. is common in bird excreta because the uric acid, xanthine, and guanine present in the urine are to a greater or lesser extent assimilated by all birds. Swinne *et al*. (1992) isolated *C. neoformans* from the residential dust of patients with HIV/AIDS and cryptococcosis-related comorbidities. This may be an indication that the presence of pigeons in places very close to the residence of these patients may contribute to the emergence of the disease. Baroni *et al*. (2006) investigated the presence of *C. neoformans* in *C. livia* excreta and air samples from church towers and surroundings over a 12-month period in Rio de Janeiro, Brazil. The findings showed that *C. neoformans* was present in all churches and in 37.8% of the 219 samples of bird excreta. Fifteen (4.9%) of the total air samples were also positive. According to Filiú *et al*. (2002) isolation of yeasts in new habitats is one of the strategies that can serve to understand the behavior of the microorganism in nature.

4 CONCLUSION

Research on fungal isolates and specifically on Cryptococcus spp. in urban areas can help in understanding the risks that yeasts pose to human health since some environmental saprophytes have become emerging pathogens, thus, increasing the number of pathogenic strains of the microorganism.

The results presented in this study demonstrate the need to alert the population, through educational campaigns, about the risks of exposure, the possibility of contracting cryptococcosis, and the importance of not feeding pigeons in leisure areas. Not feeding birds is one of the strategies for pigeon population control.

The environmental isolation of Cryptococcus spp. in public areas is important for public health, given the pigeon population growth in São Luís and the risks of exposure and propagule infection due to dispersion of the microorganism in the environment.

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