

ISOLATION OF *Cryptococcus* spp. IN EXCREMENTS OF PIGEONS (*Columba* sp.) IN THE MARINGÁ CITY, PR, BRAZIL

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ABSTRACT: The *Cryptococcus* genus is composed by 37 different species, however, the *C. gattii* and *C. neoformans* are the two most concerning species once they cause deep mycosis in humans. In this way, the present study had as aim to perform an analysis in droppings of pigeon (*Columba* sp.) from the city of Maringá, PR, in order to verify the presence of suggestive yeast forms of *Cryptococcus* spp. For this study 70 samples of pigeon excrements were evaluated which were collected in 7 different sites. These samples were submitted to the tests of thermotolerance at 37°C, urease, phenoloxidase activity and direct exam with China ink preparation. The results show that the pigeon droppings of 5,7% of the studied samples presented suggestive yeast forms of *Cryptococcus* spp. These results are relevant due to the positive samples are from the sites with a higher possibility of immunocompromised individuals flow, who are more up to have the most severe kinds of the disease caused by this fungi.

KEYWORDS: *Cryptococcus* spp; Cryptococcosis; Pigeons.

ISOLAMENTO DE *Cryptococcus* spp. EM EXCRETAS DE POMBOS (*Columba* sp.) NA CIDADE DE MARINGÁ, PR, BRASIL

RESUMO: O gênero *Cryptococcus* é composto de 37 espécies diferentes, contudo o *C. gattii* e *C. neoformans* são as mais preocupantes por causarem micose profunda em humanos. Desta forma, o presente trabalho teve como objetivo realizar uma análise em excretas de pombos (*Columba* sp.) procedentes da cidade de Maringá, PR, para verificar a presença de leveduras sugestivas de *Cryptococcus* spp. Para este estudo foram analisadas 70 amostras de excretas de pombos, coletadas em sete localidades diferentes. Essas amostras foram submetidas ao teste de termotolerância à 37°C, urease, atividade fenoloxidase e exame direto com tinta da china. Os resultados demonstraram que nos excrementos de pombos de 5,7% das localidades estudadas apresentaram leveduras sugestivas de *Cryptococcus* spp. Estes resultados mostraram-se relevantes, devido ao fato de as amostras positivas pertencerem aos locais de maior possibilidade de circulação de indivíduos imunocomprometidos, os quais estão mais propícios a contrair as formas mais graves da doença causada por este fungo.

PALAVRAS-CHAVES: *Cryptococcus* spp; Criptococose; Pombos.

Introduction

The *Cryptococcus* genus is composed by 37 different species (BIVANCO; MACHADO FILHO; MARTINS, 2006). However, the *Cryptococcus neoformans* and *Cryptococcus gattii* species are able to grow at 37°C, being able to cause disease called cryptococcosis (LUGARINI, 2007; SIDRIM; MOREIRA, 1999). However, infections caused by other *Cryptococcus* species have been reported (VIDOTTO, 2004). There are reports that other four *Cryptococcus* species (*C. albidus*, *C. laurentii*, *C. terreus* e *C. uniguttulatus*) can be found as part of the skin microbiota and occasionally causing skin damages or onychomycosis (SIDRIM; MOREIRA, 1999).

Cryptococcosis is a deep mycosis caused by the *Cryptococcus neoformans*. The *C. neoformans* is a capsulated yeast that according to their biological, ecological, epidemiological and genetic characteristics was previously classified in three varieties (*C. neoformans* var. *neoformans*; *C. neoformans* var. *grubii*; *C. neoformans* var. *gattii*) and five serotypes (A,B,C,D and AD) (DIAS *et al.*, 2006; KOBAYASHI

et al., 2005; PAPPALARDO; MELHEM, 2003). Currently two species are recognized, *C. neoformans* and *C. gattii*. The first one includes the serotypes A (var. *grubii*), D (var. *neoformans*) and AD (hybrid between var. *neoformans* and *grubii*), and the second one includes the serotypes B and C (BARONI *et al.*, 2006; CAMPBELL *et al.*, 2005; CARVALHO *et al.*, 2007; PEDROSO *et al.*, 2007).

The *neoformans* variety has a cosmopolitan distribution related to naturally soil contamination with birds droppings in urban areas, where the fungus remains viable for two years and infects a large proportion, immunocompromised individuals (FILLIU; WANKE; AGUENDA, 2002; PASSONI, 1999; REOLON; PEREZ; MEZZARI, 2004; SANTANA; COSTA; QUEIROZ, 2007; SOARES *et al.*, 2005). The *grubii* variety can be found in some healthy individuals, immunocompromised patients, veterinary sources, birds excrements, subtracts of fruits juice in brewing process, water, wood, soil and air (REOLON; PEREZ; MEZZARI, 2004). The *C. gattii* is mainly found in tropical and subtropical regions related to the ecological niche of Eucalyptus (*Eucalypt-*

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tus calmadulensis) in vegetable rests of eucalyptus in different countries, and in rural areas infecting the immunocompetent hosts (DIAZ; FELL, 2005; MACHADO; AMARAL; SEVERO, 2003; NISHIKAWA *et al.*, 2003). In this way, the agent can be isolated from pigeon droppings, perishing fruits, trees (*Eucalyptus camaldulensis*) and even sputum from healthy individuals (LACAZ *et al.*, 2002).

The yeast can survive as commensal in animals, especially in pigeons that act as natural reservoirs. These animals are protected from infection due to their high body temperature that varies from 41,5 to 43,3°C. The success of human infection is due to the virulence factors of the *C. neoformans*, which include the capability to grow at 37°C, presence of a glucuronoxylomannan (GXM) polysaccharide capsule, melanin production, secretion of proteinase and extracellular phospholipase, production of mannitol and urease (BIVANCO; MACHADO FILHO; MARTINS, 2006; DINIZ *et al.*, 2005).

Individuals are infected by inhaling microorganisms particles dispersed in the environment, in this way the entrance is the respiratory tract, leading to a primary pulmonary infection (FRIGERI; TYBUSCH; BRUN, 2001). Frequently in immunocompromised individuals and, occasionally in immunocompetent ones, the agent is spread into the blood flow damaging other organs, mainly the Central Nervous System (CNS), besides other sites such as skin, lymphonodes, bones, joints, eyes, heart, liver, spleen, kidneys, thyroid, suprarenal and even the prostate, can be regarded as reservoirs for the recurrence of the disease (BIVANCO; MACHADO FILHO; MARTINS, 2006).

Before the AIDS advent, cryptococcosis was rarely diagnosed in humans, but had global prevalence in humans, the infection is often identified in individuals with diseases that depress the immune system, such as; cancer, , autoimmune diseases, chronic hepatitis or cirrhosis and transplanted patients using immunosuppressants (LOPES *et al.*, 1997). According to the Epidemiological Bulletin, from 1980 to 2007, were 474.273 reported cases of AIDS in Brazil (Ministério da Saúde, 2007). It is estimated that 4,5% of opportunistic infections in HIV positive individuals are caused by *C. neoformans* (CALVO *et al.*, 2001), and between the years 1995 and 2005 were registered 1045 cases of cryptococcosis in the country.

The Cryptococcosis is more severe in immunocompromised, but has epidemiological relevance by being able to survive in commensalism in humans. It was related that *C. neoformans* have already been isolated from sputum, in saprophytic conditions in

oropharynges and interdigital areas of healthy individuals (LACAZ *et al.*, 2002). Based on this, it is possible to believe that asymptomatic individuals, who carry this fungus, can develop cryptococcosis if they become immunodepressed, and could also develop the disease by inhalation of great amount of propagules (REOLON; PEREZ; MEZZARI, 2004).

The present study aims to perform an analysis of pigeons droppings (*Columba sp.*) from the Maringá city, PR in order to verify the presence of *Cryptococcus spp.* suggestive.

Material and Methods

Samples collecting: The study was performed in Maringá city, PR, Brazil, which an estimated population of 300 thousand habitants, according to the Demographic Census in 2000 (IBGE). This study was performed by samples analysis of pigeons (*Columba sp.*) droppings from the Maringá city, PR. The collecting sites were selected according to the following criteria: sites inhabited by pigeons; visualization of contaminated soils with dried excrements exposed to the environment; sites with a high concentration of people, including places with the probability to find immunodepressed individuals. So the areas selected for the study were: Renato Celidônio Square, Napoleão M. da Silva Square, Raposo Tavares Square, Sete de Setembro Square, sidewalks of the Piratinin-ga Street, Neo Alves Street and Silva Jardim Street. It were collected 10 samples of each site, making a total of 70 samples for this study. The samples were removed from the soil with sterile spatulas, the aged, dried and apparently untouchable excrements of pigeons were collected and placed it into sterile plastic flask, and stored at 8 to 12°C for 24 hours

Isolation and identification: About 1,0 g of droppings were suspended in 10 mL of sterile physiological solution containing 200mg/L of chloramphenicol and 200mg/L of gentamicin. After that the samples were macerated with a sterile glass stick and mixed by vortex for three minutes, and left for 30 minutes at room temperature. Then, the second dilution was proceeded, and 0,5 mL of each supernatant was aspirated and diluted in sterile physiological solution containing 200 mg/L of chloramphenicol and 200 mg/L of gentamicin. Following, 0,1 mL of the supernatant was aspirated from the first and second dilutions and grown on niger agar containing 200 mg/L of chloramphenicol, 200mg/L of gentamicin and 100 mg/L of diphenyl. The Petri dishes were incubated at 30°C and monitored daily until the seventh day (FILIU; WANKE; AGUENDA, 2002; LUGARINI,

2007; REOLON; PEREZ; MEZZARI, 2004; SANTANA; COSTA; QUEIROZ, 2007).

The *C. neoformans* and *C. gatti* produce the enzyme phenoloxidase that catalyzes the oxidation of phenolic compounds such as tyrosine and chlorogenic acid, presented in the niger agar, producing a melanin-like pigment, giving to the colonies a dark color. Occasionally, *C. luteolus*, *C. laurentii*, *C. terreus* and the *C. albidus* can develop lighter pigmentations after prolonged incubation periods. In this way the wet, creamy, mucous aspects, smooth and dark brown colonies, were submitted to micromorphological analysis. For this, a colony from each suspects isolated was collected and homogenized in 50 µL of sterile water and 50 µL of China ink (nankin) in order to analyze the *Cryptococcus* spp. capsule by microscopy (LACAZ et al., 2002). The positive colonies for the capsule, were cultured into tubes containing Sabouraud agar with chloramphenicol (200 mg/L), gentamicin (200 mg/L) and diphenyl (100 mg/L) and cultivated at 25°C for isolation and at 37°C for thermotolerance analysis, being observed during seven days for its growing capacity (SANTANA; COSTA; QUEIROZ, 2007). Among the species of the *Cryptococcus* genus, only the *C. neoformans* and *C. gatti* are able to grow at 37°C (LUGARINI, 2007).

The isolated samples at 25°C were submitted to the urease test, by culture in tubes containing Urea-agar-Base medium and incubated at 30°C for five days. As it is a colorimetric reaction, the positive test was confirmed by change in medium color, from yellow to pink. This modification is due to the pH changing in the medium promoted by ammonia, a product of urea hydrolysis performed by *Cryptococcus* spp. urease enzyme (REOLON; PEREZ; MEZZARI, 2004; SANTANA; COSTA; QUEIROZ, 2007).

All tests were performed with negative and positive controls, that was constituted by *Cryptococcus* spp. isolated from a patient with cryptococcosis provided by the Laboratory of Mycology of the State University of Maringá (UEM).

Results

The *Cryptococcus* spp. was isolated from pigeons droppings at three different places among the seven studied in the city. Among the 70 analyzed samples; 5.7 % were positive on microscopy analysis (Figure 1), as well in testes of phenoloxidase activity, thermotolerance at 37°C and urease, suggesting the presence of *Cryptococcus* spp. in the pigeons excrements (Table 1).

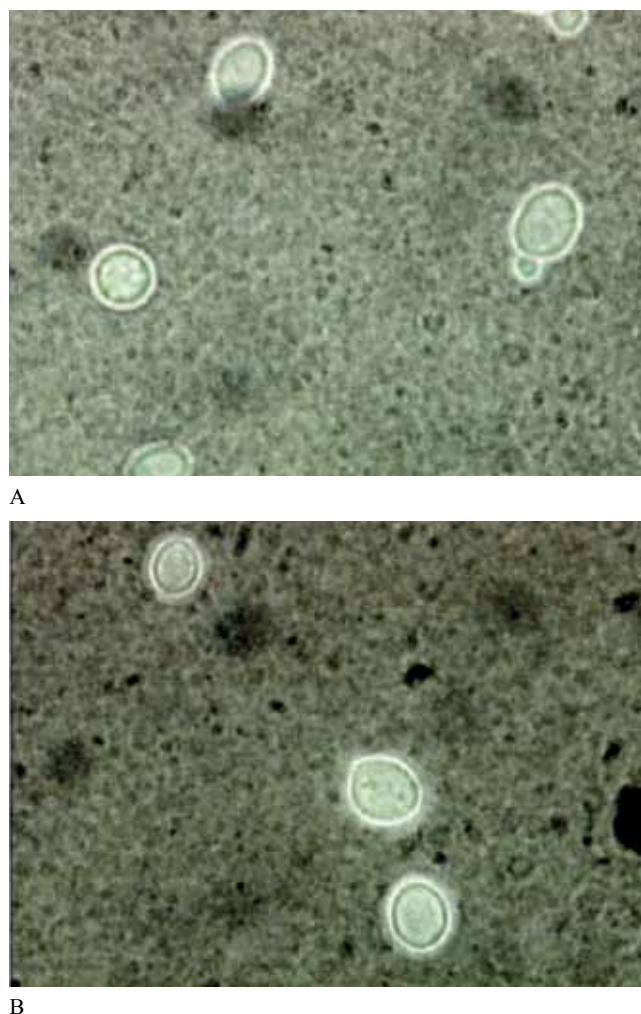


Figure 1: A: Positive control. Capsule of *Cryptococcus* spp. delineated by china ink increase of 10x. B: Sample environmental positive. Capsule of *Cryptococcus* spp. delineated by china ink increase of 10x.

Table 1: Results of tests for *Cryptococcus* spp. in pigeons excrements collected at different sites in Maringá city, PR, Brazil 2008.

Sites	Samples	China ink microscopy	Phenoloxidase activity	Thermotolerance at 37°C	Teste da uréia
Renato Celidônio Square	1 to 10	1 Positive	1 Positive	1 Positive	1 Positive
Napoleão M. da Silva Square	11 to 20	Negative	Negative	Negative	Negative
Raposo Tavares Square	21 to 30	Negative	Negative	Negative	Negative

Sete de Setembro Square	31 to 40	1 Positive	1 Positive	1 Positive	1 Positive
Piratinga Street	41 to 50	Negative	Negative	Negative	Negative
Silva Jardim Street	51 to 60	2 Positive	2 Positive	2 Positive	2 Positive
Néo Alves Street	61 to 70	Negative	Negative	Negative	Negative

Discussion

Our studies suggested, for the first time, the presence of yeasts suspect for *Cryptococcus* spp. in droppings of pigeons from Maringá-PR. The ecological relation of the *C. neoformans* was first described by Emmons et al (1995), through the isolation of the yeast from pigeons excrements in the United States. After that, many studies were carried out worldwide confirming this finding (SANTANA; COSTA; QUEIROZ, 2007). In Brazil, in the city of Rio de Janeiro, 824 samples of dust, soil and birds droppings in domestic environment were examined, Passoni et al (1998) which showed the presence of the *C. neoformans* var. *neoformans* as more related with the birds droppings, mainly the pigeons.

In the Maringá city, among the positive sites, the Sete de Setembro Square and the Silva Jardim Street were the sites with a higher probability to find immunodeficient individuals, once in these areas there is a large concentration of hospitals, laboratories and medical clinics. The Renato Celidônio Square, where it was also isolated, is a site of frequent events and for its characteristics also has high concentration of children and elderly people. According to Kobayashi et al (2005) it has been postulated that the predominance of *C. neoformans* var. *neoformans* in patients with AIDS is due to the higher exhibition of individuals to the natural habitat of this fungus.

However, Reolon; Perez; Mezzari (2004) reports that in these places the fungus can be spread on the air and, when inhaled in great amounts it could also lead the disease in immunocompetent individuals. Besides it, Lacaz et al (2002) reports the presence of *Cryptococcus* spp. in an ecological relation of commensalism with healthy individuals.

In Brazil, it was reported 13.071 cases of AIDS. The incidence rate of AIDS in young people from 13 to 24 years in Paraná State is 12,0/100.000 habitants. In Maringá, 22 cases of HIV-positive individuals were notified in 2007 (Ministério da Saúde, 2007). When these data were observed and considering about the estimated that 4.5% of the opportunistic infections have been caused by the *C. neoformans* (CALVO et al., 2001), it is noticed the importance and the need of ecoepidemiological studies to determine the prevalence of infectious agent in the envi-

ronment, once that three out of the seven sites studied presented positive isolated *Cryptococcus* spp.

Baroni et al (2006) reports the influence of temperature in the proliferation and survivor of the *Cryptococcus* spp., in which high summer temperatures can inhibit the growing of this yeast or even inactivate it. This fact might contribute to explain the number of isolation in Maringá-PR, once these samples were collected in high temperature period between February and April, 2008. However, it was a rainy period, and in the wet droppings, the bacterial decomposition causes a strong alkalization of the substrate and growing inhibition of the *C. neoformans* (KWONG-CHUNG; BENNETT, 1992). Due to it, it was set a period of 10 days without rains to collect the dried droppings.

Findings of other researchers have demonstrated that the capsules of environmental isolated are thinner than the ones observed in patients, and culture cells lost is stickiness and after successive cultures the microorganisms present a small capsular envoltorium (CALVO et al., 2001; LACAZ et al., 2002; LUGARINI, 2007). In this way, the presented results, demonstrate that some samples, stained with China ink and analyzed under a microscopic there is evidence of capsules with a smaller diameter.

During the culture of the samples it was possible to observe the difficulty to isolate the *Cryptococcus* spp. due to the presence of fungus and contaminant bacteria, even with adding antibacterial and antifungal agents to the culture. This difficulty can be due to the samples, once they are naturally contaminated, and also due to the culture medium which is rich in nutrients that make possible the growing of many others fungus and bacteria (PAL, 1997). These circumstances make impossible to count the *Cryptococcus* spp. colonies, mainly when the respective content were too low; and also due to the effects of the dilutions which is probable to exclude the yeast that are the minority (BERNARDO; MARTINS; MARTINS et al., 2001).

In a month, the colonies, for their mucous texture and aspect of condensed milk, flowed on the surface and accumulated in the bottom of the tubes, characterizing one more aspect referred to the presence of the *Cryptococcus* spp. in the positive samples (LACAZ et al., 2002).

Conclusion

This study revealed the presence of yeasts suspect for *Cryptococcus* spp. in droppings of pigeons from Maringá-PR. These results bring a warning to the city of Maringá-PR, in order to minimize the risk of exhibition to micro focus of *Cryptococcus* spp. In site of public flow and near a higher concentration of people, through supervision of the conditions of hygiene and cleaning of the droppings, awareness of the risky sites, as well as reducing the alimentary abundance for the pigeons, in order to not attracting them to these sites of higher epidemiological risk. The presence of the genus *Cryptococcus* in samples analyzed in this study, suggest that subsequent studies should be carried out in order to determine the species and their variety as well as to establish eco-epidemiology of this agent in the city of Maringá-PR.

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