

Cryptococcus albidus var. *albidus* Isolated from Turquoise-Fronted Parrots (*Amazona aestiva*: Psittacidae) Kept in Captivity: A Probable Reservoir Ecological of Fungal Specimen

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Abstract

Cryptococcus is an opportunistic yeast that causes life-threatening infections as meningoencephalitis primarily in immunocompromised hosts, generally associated with AIDS. The source of this organism is mainly pigeon excreta; however, other avian species' excreta are implicated as a source of this yeast. The aim of this study was to perform the isolation of yeasts of the *Cryptococcus* genus from the cloacae of 40 parrots kept in captivity area of the genus *Amazona aestiva*. These birds were anesthetized, the cloacae washed, and then swabs from the cloaca collected. The yeasts isolated from cloacae birds were studied by phenotypic and genotypic methods. The production of extracellular enzymes as virulence factors (protease and phospholipase) was performed too. From the total of parrots studied, 10 strains of yeasts were isolated. Nine of the strains belonged to the specie *Cryptococcus albidus* var. *albidus* and one sample belonged to the specie *Cryptococcus laurentii*. The extracellular enzymes research demonstrated that 80% of the isolates were phospholipase producers and all of them were protease positives. These results suggest that not just the environment but also the birds of *A. aestiva* genus may be the carriers of *C. albidus*. We point out that the strains produced virulence factors. This is the first report of the isolation of *C. albidus* var. *albidus* of *A. aestiva* parrots and to assert that this bird is a special ecological niche of capped yeast.

Keywords: Yeasts; Psittacidae; *Cryptococcus albidus*; Virulence factors

Introduction

Cryptococcosis is one of the leading community-acquired opportunistic mycoses and is associated with high mortality and morbidity. The major predisposing factors are cellular immune defects like those affecting patients with AIDS, with malignancies, or receiving corticosteroid or immunosuppressive therapy [1-3]. This disease is the great relevance in the medical mycology [4,5].

The yeasts of the complex *Cryptococcus* present 70 species and most of them are related to the environment. From these, *Cryptococcus neoformans* and *Cryptococcus gattii* are considered pathogenic [6]. *Cryptococcus albidus* and *C. laurentii* are being clinically highlighted due to the increase of the frequency. *Cryptococcus albidus* already been isolated from blood, lung, skin, and cerebrospinal fluid [7].

It is possible to verify that several studies evidencing the importance of bird dropping as a suitable substrate for the growth of yeasts and filamentous fungi [8-15]. Birds easily conduct the agent in the environment through its feces and become a zoonotic problem due to its proximity with the humans, explained by the fact that it is a peridomestic animal. However, little is known about the existence of *C. albidus* var. *albidus* in wild birds and from captivity.

Many reports related the association of the *Cryptococcus* with the environment as well as the correlation of them with animals mainly with birds like pigeons. Pigeons conduct *C. neoformans* in the environment through their faeces and become a zoonotic problem due to their

proximity with the humans. The literature is rare about the presence of *Cryptococcus* complex in wild and captivity birds [10,11,13,14].

Brazil has one of the largest bird species in the world, with endemic species, has an undeniable importance in the global biodiversity scenario [16], which makes the country one of the most important in diversity and conservation [17,18]. Psittacids have a great representation in the country, of which 23 are endemic and occur only in Brazil [18,19]. The group is known for large macaws, beautiful parrots capable of mimicking human voices and colorful parakeets [17].

Amazona aestiva, which is commonly called Turquoise-fronted parrot is a bird of the family *Psittacidae*. This bird has approximately 38 cm, weighs about 400 grams and its life expectancy is 80 years. Their nests are found in the cavities of trees and fruit bushes. It is found in Bolivia, Paraguay and northern of Argentina. In Brazil, this bird is more

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frequent in the North and Northeast regions, but can still be observed in the midwest, south-east and south regions of the country, in the humid forests, in rivers, and closed borders. This species is the most popular of the Psittacidae family for being sociable, for scoring imitate human sounds and be easily found [18,19].

Since 1990, *A. aestiva* is being seen in São Paulo city, probably from escapes captivity and migration to large urban centers. This migration was due to deforestation resulting from the lack of food for these kites [20]. Possibly the most popular of all Brazilian birds and one of the biggest targets of the illegal trade of wild animals in the world [16].

The aim of this study was to perform the isolation of yeasts belonging to the genus *Cryptococcus* from cloacae of parrots of the genus *Amazona aestiva*, as well as to perform the phenotypic and genotypic identification and investigation of presence of the virulence factors (protease and phospholipase).

Materials and Methods

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (CEUA/127).

The collected materials of cloacae were originated from 40 parrots of the genus *A. aestiva* without age or gender defined. These birds are captivity in a private captivity located in the São Paulo, Brazil. The parrots used in this study did not present any visible sign of disease. The parrots captivity had two separated environments; the indoor area, with many closed enclosures, and the outdoor area, that was an open place delimited by fences with some places protect of rain and wind.

Collecting material

The analyzed material was collected from two regions of the birds: crops and cloacae. The birds were anesthetized to perform this procedures and before collecting the material from the crop, the birds have it washed with 5 mL of physiological saline 0.9% sterile through the introduction of a urethral sound sterile number 5 or 6. The sounder was introduced initially in the oral cavity of the bird towards the esophagus until the crop. Before collecting the material from cloacae, it was previously cleaned with 70% alcohol. To obtain samples of the cloacae, was used swab moistened with physiological saline (0.9%) being kept refrigerated until the inoculation on Petri dish with Sabouraud dextrose agar (Difco, USA) with chloramphenicol 0.25%. The samples were performed in triplicate and incubated from 7 to 15 days at 30°C. All the yeasts samples were isolated in tubes containing the Sabouraud dextrose agar (Difco, USA) with chloramphenicol 0.25%. Morphological and physiological tests were did according to Kurtzman [6].

Identification of yeasts

The strains were identified according the manual the yeasts it may be described: the India ink test to verify the morphology of the yeast as well as the formation or not of the polysaccharide capsule; test of urease production in which the yeasts were inoculated in Christensen medium to verify the production of enzymes by the yeast. Other tests were performed as growth in medium canavanine glycine bromothymol (CGB) to observed blue cobalt colour; phenol oxidase production in a medium containing dopamine (dark brow-black

colour). Additional tests were realized to identify the three varieties of *C. albidus*: assimilation of maltose, melizitose, L-tartrate, L-lysine; growth at 35°C; growth at yeast extract agar (Difco, USA) to observed the colour of the strains and starch production [6].

The species identification was confirmed by polymerase chain reaction (PCR) and sequencing of ITS region, employing primers pairs ITS1/ITS2. Sequencing reactions were performed in automatic sequencer 3130 Genetic Analyzer (Life Technologies, CA, USA), by capillary electrophoresis, employing the Kit Big Dye Terminator Cycle Sequencing V3.1 (Life Technologies, CA, USA), and analyzed by the Sequencing Analysis v 5.3.1 and SeqScape v 2.6. Sequencing was performed in order to confirm the identity of the amplicons with sequences from the database of NCBI GenBank.

All the experiments followed standards strains *C. gattii* ATCC 56990 e *C. neoformans* ATCC 90112.

The production of phospholipase and proteinase were also investigated. In the first test, the strains were inoculated in a phospholipase agar medium with yolk and incubated at 25°C up until 15 days [21]. In the test to verify the protease production, the strains were inoculated on a medium containing bovine albumin fraction V (Sigma, EUA) and incubated at 25°C up until 15 days [22]. The value of the enzymatic activity (PZ) was obtained by calculating the ratio between the diameters of the colony and the one zone by the colony and the precipitation/degradation zone. The results of the extracellular enzyme production were analyzed according to the pattern described by Price et al. and Rüchel et al. [21,22]. A Pz value of 1.0 mm represent the absence of enzymatic activity while values between 0.64 mm and 1.0 mm reflect positive activity and with values less than 0.64 mm suggesting strongly positive enzymatic activity.

Statistical analysis was performed to assess the significance or absence of the number of isolated indoor and outdoor area (Test qui-square- $P < 0.05$).

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethical approval protocol CEUA/127, Institute of Biomedical Sciences/University of São Paulo, adopted on 29 November 2011).

Results

The 40 birds studied, yeasts belonging to the complex *Cryptococcus* were isolated from 10 birds (25%). The majority of them (90%) were identified as *Cryptococcus albidus* (Saito) CE Skinner and one as *Cryptococcus laurentii* (Kufferath) CE Skinner.

The isolates that grew on yeast extract agar presented color cream to pinkish-cream and smooth surface, being that one strain presented pale grayish-cream color and mucous aspect. All the strains presented capsule or incipient capsule.

The growth on CGB medium all the yeasts did not demonstrated blue-cobalt colour and one strain *C. laurentii* revealed light blue color. On medium containing dopamime, all strains presented a light yellow colour. These tests are positive to *C. neoformans* or to *C. gattii*, blue cobalt colour and dark brown colour, respectively.

Additional tests revealed that the variety of *C. albidus* was var. *albidus*: assimilation positive for maltose, melizitose, L-tartrate and

L-lysine; growth at yeast extract agar and with yellow color; growth at 35°C; and starch production.

According Kurtzman et al. nine strains belong to *C. albidus* var. *albidus* and only one strain belong to the specie *C. laurentii* [6]. Through the analysis of the ITS region nine of the samples were identified as *C. albidus* (99-100% identity) and one as *C. laurentii*, (99% identity).

The phospholipase production was observed in 80% of the strains as high producers and one strain of *C. laurentii* and one strain of *C. albidus* not produce this enzyme. Similar results could be observed in protease production because the strains were high producers in 80% and 20% were only producers.

Among the strains identified as *Cryptococcus*, 6 (60%) were originated from birds that were found in the indoor area and 4 (40%) were originated from animals of the outdoor area as it is shown in the Table 1. There was no significant difference between the isolation in indoor / outdoor areas (Test qui-square- $P < 0.05$).

Discussion

The relation *Cryptococcus*-bird has ecological and epidemiological relevancy and widely studied. There are a great number of publications about this topic. From the first description of the isolation of the yeast from the pigeon's excretas by Emmons [23], many other researches were published highlighting the prevalence of the fungus in the faeces of columbiformes and other birds [24], in different places around the world. Carter and Baroni et al. linked the presence of the yeast with nests and chicks [10,25].

The isolation of yeasts belonging to the genus *Cryptococcus* is frequent when related to organic material principally from bird's faeces. However, the recuperation of the samples belonging to this genus, from the cloacae of Psittacidae, object of this study, is a factor still little studied.

The difficulty of the isolation of the agent may also observed in many studies, and some factors that influence these isolations are growth of filamentous fungi, temperature changes after taking the sample to arrive at the laboratory, and humidity [26-29]. This fact occurs because dried bird droppings have fertility characteristics for fungal species growth due to high concentrations of nitrogenous bases and, as they become old, they contain higher concentrations of fungi than when they have recently been eliminated [14,15,30].

In this present study, the parrots had shown being capable of lodging *C. albidus* var. *albidus* in the cloacae and may disseminate it in the environment through the faeces. Other studies also isolated yeasts from the parrots but not from *A. aestiva* genus [31,32]. It is possible to verify many reports that describe the link of yeasts belonging to the complex *Cryptococcus* with the environment as well as the correlation of them with the animals. A retrospective of scientific studies related birds and yeasts in different countries and the geographical distribution of etiological agents can be observed and analyzed in this study (Table 2).

It is relevant to mention that the *C. albidus* has been reported in various clinical materials and causing diverse infections, mainly in immunocompromised patients [42,43]. Few cases of animal *C. albidus* infections have also been reported [44]. It should be emphasized that in this study none of the birds had any clinical manifestation.

The major number of researches are restricted to species *C. neoformans* and *C. gattii* and their relation with the environment. Other species of the genus as *C. albidus* and *C. laurentii* are less described

without considering their relevance as pathogens and their interactions with the environment. In a study performed with samples originated from the cloacae of Psittacidae, González-Hein et al. isolated strains of *C. albidus* and *C. laurentii* [45]. However, this study did not refer to the genus *A. aestiva*. In our study, the isolated strains were phenotypically and biochemically identified as *C. albidus* var. *albidus* and *C. laurentii*, being that the first was recovered in nine of the ten carrier parrots.

Other varieties of *C. albidus* (*C. albidus* var. *ovalis* and *C. albidus* var. *kuetzinguii*) do not produce starch and the variety *C. kuetsinguii* does not assimilate maltose or melezitose, tests that are positive for *C. albidus* var. *albidus*. *Cryptococcus albidus* var. *kuetzinguii* were only isolated in Europe from environmental sources [46] and *C. albidus* var. *ovalis* was obtained from goat dung in Pakistan. The geographic distribution of *C. albidus* var. *albidus* is wide [6].

All isolates of *C. albidus* var. *albidus* produced phospholipase and protease at a high level. A special clinical relevance is point out because of the fact that these enzymes are factors of virulence [47,48].

Cryptococcus albidus has been reported as an antagonist of certain postharvest pathogenic fungi [49]. This fact makes us believe that in these *A. aestiva* faeces studied, the unike presence of *C. albidus* may have inhibited the growth of other fungal species such as *C. neoformans*. The *C. albidus* strains are less susceptible to azole antifungals, important factor, but it is sensitive to amphotericin B [6].

Slide agglutination experiments with strains of *C. neoformans* factor shown that antigenic formulae of *C. albidus* strains were similar to serotype A of *C. neoformans* [50]. These findings reveal the antigenic proximity of *C. albidus* with *C. neoformans* serotype A.

The results obtained in this research confirm the role and the relevance of the parrots from the genus *A. aestiva* as a source of dissemination of yeasts in the environment. Most of the yeasts were producers of the extracellular enzymes proteinase and phospholipase, considered as virulence factors of microorganisms. By the literature and by the interesting results of this research, the specie *C. albidus* should be better studied referring to the epidemiology and interaction *C. albidus*-environment-human.

Apparently birds seen as healthy can act as hosts for pathogenic microorganisms to defecate and contaminate the environment with several yeast genera that present zoonotic potential, so it is important to mention the relevance of avian zoonosis because these are infections that often remain asymptomatic in birds, misdiagnosed as healthy, making it difficult to determine the correct diagnosis and subsequent treatment, thus increasing the chances of transmission to bird keepers, zoo visitors, pet store workers, and pet owners.

Birds	Area	Identification
2	Indoor, n° 15	<i>C. albidus</i> var. <i>albidus</i>
3	Indoor, n° 15	<i>C. albidus</i> var. <i>albidus</i>
15	Indoor, n° 03	<i>C. albidus</i> var. <i>albidus</i>
16	Indoor, n° 10	<i>C. albidus</i> var. <i>albidus</i>
19	Indoor, n° 12	<i>C. albidus</i> var. <i>albidus</i>
21	Indoor, n° 13	<i>C. albidus</i> var. <i>albidus</i>
26	Outdoor	<i>C. laurentii</i>
29	Outdoor	<i>C. albidus</i> var. <i>albidus</i>
31	Outdoor	<i>C. albidus</i> var. <i>albidus</i>
34	Outdoor	<i>C. albidus</i> var. <i>albidus</i>

Table 1: Relation of the birds, local of isolation and identification of the strains and yeasts originating from parrots of the genus *A. aestiva*.

Authors/reference	City/Countries	(N) Species birds studied	Patology/Clinical signs and Substrate	Etiological agent	Prevalent agent
Filiú et al. [12]	Mato Grosso do Sul/Brazil	(20) Passeriformes, Psittaciformes, Columbiformes.	Faecal samples	<i>Cryptococcus</i> spp.	<i>Cryptococcus neoformans</i>
Malik et al. [33]	Sydney/Australia	(15) <i>Psittacus erithacus</i> , <i>Eclectus roratu</i> , <i>Alisterus scapulari</i> , <i>Aptery australis</i> , <i>Cacatua galerita</i> , <i>C. tenuirostris</i> , <i>C. roseicapilla</i> and <i>Columba livia</i>	Infection respiratory tract and lesions/cytological and histological preparations	<i>Cryptococcus</i> spp.	<i>Cryptococcus grubii</i> and <i>C. bacillisporus</i>
Raso et al. [34]	São Paulo/Brazil	(7). <i>Chamosyna papou</i> , <i>Lorius lorry</i> , <i>Trichoglossus glodiei</i> , <i>Psittacula krameri</i> , <i>psittacul erithacus</i>	Incoordination paralysis and superficial lesions/Faeces, organ fragments and aspirated biopsy samples	<i>Cryptococcus</i> spp.	<i>Cryptococcus gattii</i>
Abegg et al. [35]	Rio Grande do Sul/Brazil	(38). Obtained from psittaciformes: Psittacidae, Cacatuidae and Psittacula.	Faecal samples	<i>Cryptococcus</i> spp.	<i>Cryptococcus grubii</i> <i>Cryptococcus gattii</i>
Cafarchia et al. [36]	Bari/Italy	(1.721) migratory and caught birds	Cloacae samples	<i>Candida</i> spp., <i>Cryptococcus</i> spp., <i>Rhodotorula</i> spp.	<i>Rhodotorula rubra</i> and <i>Cryptococcus albidus</i>
Cafarchia et al. [37]	Bari/Italy	(768) Obtained birds of prey, Passeriformes, migratory birds and aviaries birds.	Cloacae samples	<i>Candida</i> spp., <i>Cryptococcus</i> spp., <i>Trichosporon</i> spp., <i>Rhodotorula</i> spp., and <i>Saccharomyces</i> spp.	<i>Candida albicans</i> , <i>Cryptococcus albidus</i> , <i>C. laurentii</i> , <i>C. neoformans</i> .
Lugarini et al. [38]	Paraná/Brazil	(141) Passeriformes and Psittaciformes	Faecal samples	<i>Cryptococcus</i> spp.	<i>Cryptococcus grubii</i> <i>Cryptococcus gattii</i>
Santos et al. [29]	Paraná/Brazil	(29). <i>Amazona aestiva</i> , <i>Aratinga solstitialis</i> , <i>Nymphicus hollandicus</i>	breathing problems/ oral cavity/ cloacae samples	<i>Candida</i> spp. <i>Cryptococcus</i> spp.	<i>Cryptococcus neoformans</i>
Brilhante et al.	Ceará/Brazil	(60) <i>Nymphicus hollandicus</i>	Oral cavity, crop and cloacae samples	<i>Candida</i> spp., <i>Rhodotorula</i> spp. and <i>Trichosporon</i> spp.	<i>Candida albicans</i>
González-Hein et al. [39]	Santiago/Chile	(113) Captive species of birds	Droppings samples	<i>Cryptococcus</i> spp.	<i>Cryptococcus albidus</i> , <i>C. neoformans</i> and <i>C. uniguttulatus</i> .
Marinho et al.	São Paulo/Brazil	(36) Passeriformes (passerines)	Droppings samples	<i>Penicillium</i> spp., <i>Aspergillus</i> spp., <i>Candida</i> spp., <i>Trichosporon</i> spp. and <i>Cryptococcus</i> spp.	<i>Penicillium</i> spp., <i>Cryptococcus gattii</i> and <i>C. neoformans</i>
Mendes et al. [15]	Rio Grande do Sul/Brazil	(50) Different species of birds	Faecal samples	<i>Malassezia</i> spp., <i>Candida</i> spp., <i>Trichosporon</i> spp., <i>Geotrichum</i> spp., <i>Aspergillus</i> spp., <i>Penicillium</i> spp. and <i>Cryptococcus</i> spp.	<i>Candida albicans</i> <i>Cryptococcus laurentii</i>
Dynowska et al. [40]	Warnia/Poland	(450) Charadriiformes (seagulls)	Cloacae samples	<i>Candida</i> spp., <i>Cryptococcus</i> spp. and <i>Rhodotorula</i> spp.	<i>Candida albicans</i> <i>Cryptococcus satoii</i> , <i>C. neoformans</i> .
Abbas et al. [41]	Baghdad/Iraq	(100) Birds: pigeon, pet bird and chicken.	Faecal samples	Moulds and Yeasts	<i>Penicillium</i> spp. <i>Cryptococcus neoformans</i>
*Simi et al. (personal communication – Out/2017)	Mato Grosso/Brazil	(149) Birds of prey and Psittaciformes.	Faecal samples	Moulds and yeasts	<i>Aspergillus niger</i> , <i>Candida kefir</i> and <i>Cryptococcus albidus</i>
**Nascimento et al.	São Paulo/Brazil	(40) <i>Amazona aestiva</i>	Cloacae samples	<i>Cryptococcus</i> spp.	<i>Cryptococcus albidus</i> and <i>C. laurentii</i>

Table 2: Geographic distribution of yeasts that cause infection in birds, etiological agents and their dominant pathogens in the study areas.

Conclusion

More studies involving wild birds as *A. aestiva* are necessary because they could be reservoirs and disseminators of these yeasts in the environment. This is the first research about the isolation of *C. albidus* var. *albidus* from the cloacae of parrots of the genus *A. aestiva* in Brazil.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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