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# Yeast microbiota of raptors: a possible tool for environmental monitoring

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## Summary

**Twenty-two raptors from a rehabilitation centre were evaluated for the presence of yeasts prior to returning them to the wild, and the recovered *Candida* isolates were tested for *in vitro* antifungal susceptibility and phospholipase production. Samples were collected from the crop/lower esophagus and cloaca. *In vitro* antifungal susceptibility and phospholipase production of 21 *Candida* strains were assessed through broth microdilution and growth on egg yolk agar respectively. Twenty-seven isolates, belonging to seven species, were recovered from 16 tested birds, with *C. albicans* and *C. famata* as the most prevalent species. Three out of 21 isolates (2 *C. albicans* and 1 *C. tropicalis*) were simultaneously resistant to fluconazole and itraconazole. As for phospholipase production, 8 (8/21) isolates (6 *C. albicans*, 1 *C. famata* and 1 *C. parapsilosis*) showed enzymatic activity. The most relevant finding in this study was the isolation of resistant *Candida* spp. from wild raptors that had never been submitted to antifungal therapy, which suggests exposure to environmental contaminants. Based on this, we propose the assessment of *Candida* spp. from the gastrointestinal tract of raptors as a tool for environmental monitoring.**

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## Introduction

Raptors belong to the avian orders *Ciconiiformes* and *Strigiformes*, which include eagles, hawks, owls and vultures (ITIS, 2011). They are commonly found in Brazilian wild animal rehabilitation centres, mainly, due to trauma-related health disorders and illegal trade (ICMBio, 2008).

According to Brazilian legislation, before releasing birds into their natural environments, they should be screened for some pathogens, including *Candida* spp. and *Cryptococcus* spp. Such procedures are routinely performed at the Triage Center for Wild Animals (CETAS) of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), where injured or seized wild animals are kept until they are rehabilitated and fit to be released. In this study, 22 raptors from CETAS were evaluated for the presence of yeasts, prior to returning them to the wild, and the recovered *Candida* isolates were tested for *in vitro* antifungal susceptibility and phospholipase production.

## Results and discussion

Twenty-two raptors, belonging to seven different species, were assessed: 8 roadside hawks (*Rupornis magnirostris*), 6 savanna hawks (*Heterospizias meridionalis*) 4 caracaras (*Caracara plancus*), 1 American black vulture (*Coragyps atratus*), 1 yellow-headed caracara (*Milvago chimachima*), 1 Harris's hawk (*Parabuteo unicinctus*) and 1 barn owl (*Tyto alba*). The specimens *M. chimachima*, *P. unicinctus* and *T. alba* had been captured for illegal trade and were seized by IBAMA and taken to the triage centre (CETAS), where they were kept for a long period of time. The other specimens were taken from the wild to CETAS for presenting trauma-related disorders, where they spent a short period of time because they were released as soon as they recovered. While in captivity, they were maintained in collective enclosures and their diet mainly consisted of beef supplemented with minerals and vitamins. This study was approved (protocol number 02/09) by the Animal Research Ethics Committee of the Federal University of Ceará.

**Table 1.** Yeast isolated from the gastrointestinal tract of raptors and *in vitro* antifungal susceptibility and phospholipase production of the recovered *Candida* spp.

| Bird species | Positive /tested birds | Collection site | Yeast species | Minimum inhibitory concentration (μg ml$^{-1}$) | | | Phospholipase production |
|---|---|---|---|---|---|---|---|
| | | | | Amphotericin B | Fluconazole | Itraconazole | |
| *Caracara plancus* | 3/4 | Crop/lower esophagus | *T. asteroides* (1)[a] | – | – | – | – |
| | | | *R. mucilaginosa* (1) | – | – | – | – |
| | | Cloaca | *C. albicans* (1) | 0.5 | 4 | 0.5 | 0.36 |
| | | | *C. famata* (1) | 0.25 | 1 | 0.03125 | 1 |
| *Coragyps. atratus* | 1/1 | Cloaca | *C. albicans* (1) | 0.125 | > 64[b] | > 16[b] | 0.53 |
| *Heterospizias meridionalis* | 3/6 | Crop/lower esophagus | *C. albicans* (1) | 0.5 | 0.25 | 0.03125 | 1 |
| | | | *C. famata* (1) | 0.25 | 1 | 0.0625 | 0.68 |
| | | | *C. parapsilosis* (1) | 0.25 | 0.25 | 0.0625 | 0.97 |
| | | | *R. mucilaginosa* (1) | – | – | – | – |
| | | Cloaca | *C. parapsilosis* (1) | 0.125 | 4 | 0.03125 | 1 |
| *Milvago chimachima* | 1/1 | Cloaca | *C. albicans* (1) | 0.25 | 1 | 0.03125 | 0.42 |
| | | | *C. famata* (1) | – | – | – | 1 |
| *Parabuteo unicinctus* | 1/1 | Crop/lower esophagus | *C. famata* (1) | 0.25 | 1 | 0.0625 | 1 |
| *Rupornis magnirostris* | 6/8 | Crop/lower esophagus | *C. albicans* (3) | 0.5–1 | 0.5–> 64[b] | 0.125–> 16[b] | 0.47, 0.55, 1 |
| | | | *C. famata* (3) | 0.125–0.5 | 0.5–16 | 0.0625–0.125 | 1 |
| | | | *C. tropicalis* (2) | 0.03125–0.5 | 1–> 64[b] | 0.125–> 16[b] | 1 |
| | | | *R. mucilaginosa* (1) | – | – | – | – |
| | | Cloaca | *C. albicans* (1) | 0.5 | 0.5 | 0.03125 | 0.39 |
| | | | *C. catenulata* (1) | – | – | – | 1 |
| | | | *C. parapsilosis* (1) | 0.5 | 0.5 | 0.03125 | 1 |
| | | | *C. tropicalis*(1) | 0.25 | 1 | 0.125 | 1 |
| *T. alba* | 1/1 | Cloaca | *C. famata* (1) | 1 | 8 | 0.03125 | 1 |

**a.** Number of recovered isolates.
**b.** Resistant isolates.

Prior to specimen collection, the birds were clinically evaluated (Brilhante *et al*., 2010). Only healthy birds were tested. Samples were collected from the crop/lower esophagus and cloaca and processed on birdseed (*Guizotia abyssinica*) agar supplemented with chloramphenicol (0.5 g l$^{-1}$) and biphenyl (0.1%), at the Specialized Medical Mycology Center of Federal University of Ceará, Brazil, as previously described (Brilhante *et al*., 2010). Recovered colonies were identified through micromorphological and biochemical parameters. VITEK 2® (bioMérieux, USA) was used to confirm dubious identification (Brilhante *et al*., 2010).

Sixteen out of the 22 evaluated birds (72.73%; 6/8 *R. magnirostris*, 3/6 *H. meridionalis*, 3/4 *C. plancus*, 1/1 *C. atratus*, 1/1 *M. chimachima*, 1/1 *P. unicinctus* and 1/1 *T. alba*) were positive for the presence of yeasts (Table 1). Yeasts were recovered from crop/lower esophagus, cloaca or both anatomical sites of eight (50%), five (31.25%) and three (18.75%) birds respectively.

A total of 27 isolates were obtained, with *C. albicans* ($n = 8$) and *C. famata* ($n = 8$) as the most prevalent ones, followed by *C. parapsilosis* ($n = 3$), *C. tropicalis* ($n = 3$), *Rhodotorula mucilaginosa* ($n = 3$), *C. catenulata* ($n = 1$) and *Trichosporon asteroides* ($n = 1$) (Table 1). Four individuals simultaneously presented two yeast species in the crop, which were *C. parapsilosis* and *R. mucilaginosa* from one *H. meridionalis* and *C. albicans* and *C. famata*; *C. albicans* and *C. tropicalis* or *C. famata* and *R mucilaginosa* from three *R. magnirostris*. In addition, two yeast species (*C. albicans* and *C. famata*) were simultaneously isolated from the cloaca of two birds (1 *C. plancus* and 1 *M. chimachima*) and only one bird (*R. magnirostris*) presented two yeast species in both anatomical sites (*C. famata* and *C. tropicalis* in the crop; *C. catenulata* and *C. tropicalis* in the cloaca). This last animal was the only one from which one species (*C. tropicalis*) was recovered from both evaluated sites.

Even though it is reported that the composition of the gastrointestinal yeast microbiota varies according to species-specific aspects, such as diet (Cafarchia *et al*., 2006a), in reality, it does not seem to vary greatly, considering that the yeast species recovered in this study were similar among the evaluated bird species and to other reports concerning other avian groups, such as psittacines (Vieira and Acqua-Coutinho, 2009; Brilhante *et al*., 2010) and ostriches (Melville *et al*., 2004). Unlike what was expected, based on previous reports (Cafarchia *et al*., 2006b), *Cryptococcus* spp. isolates were not recovered.

Twenty-one *Candida* isolates were submitted to antifungal susceptibility test: 8 *C. albicans* (4 from the crop/lower esophagus and 4 from the cloaca); 7 *C. famata* (5 from the crop/lower esophagus and 2 from the cloaca); 3 *C. parapsilosis* (1 from the crop/lower esophagus and 2 from the cloaca) and 3 *C. tropicalis* (2 from the crop/lower esophagus and 1 from the cloaca).

The antifungal MICs for these microorganisms were determined through broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute, document M27-A3. The microdilution plates were read after 24 and 48 h of incubation at 35°C, but the MIC values considered were those obtained after 48 h of growth. MICs for fluconazole (Pfizer, Brazil) and itraconazole (Janssen Pharmaceutica, Belgium) were defined as the lowest drug concentration capable of inhibiting 50% of growth when compared with the growth control well, and for amphotericin B (Sigma Chemical Corporation, USA), it was defined as the lowest concentration at which no growth was observed. MICs of $> 1$, $\geq 1$ and $\geq 64$ μg ml$^{-1}$ indicated resistance to amphotericin B, itraconazole and fluconazole respectively (CLSI, 2008; Sidrim *et al*., 2010; Brilhante *et al*., 2011). However, for *C. albicans*, *C. parapsilosis* and *C. tropicalis* the considered breakpoint for fluconazole was $\geq 8$ μg ml$^{-1}$ (Pfaller *et al*., 2010). All strains were tested in duplicate and the assay was repeated, when resistance was detected, in order to confirm the results.

All *Candida* isolates were screened for phospholipase production, on egg yolk agar, as previously described (Price *et al*., 1982). Phospholipase activity (Pz) was determined by calculating the ratio between the diameter of the fungal colony and the total diameter, including the colony and the precipitation zone. Pz = 1 indicated negativity for phospholipase production; Pz<1 indicated positivity for phospholipase activity and Pz<0.64 indicated strong enzymatic activity (Price *et al*., 1982; Sidrim *et al*., 2010; Brilhante *et al*., 2011).

MICs for amphotericin B ranged from 0.031 to 1 μg ml$^{-1}$ (MIC50 = 0.25 μg ml$^{-1}$; MIC90 = 1 μg ml$^{-1}$). For fluconazole and itraconazole, MICs varied from 0.125 to $> 64$ μg ml$^{-1}$ (MIC50 = 1 μg ml$^{-1}$; MIC90 $\geq 64$ μg ml$^{-1}$) and from 0.031 to $> 16$ μg ml$^{-1}$ (MIC50 = 0.0625 μg ml$^{-1}$; MIC90 $\geq 16$ μg ml$^{-1}$) respectively (Table 1). Three isolates (3/21) (two *C. albicans* and one *C. tropicalis*) exhibited *in vitro* antifungal resistance and were resistant to both azole derivatives.

Parallely, these resistant strains were tested against promethazine, which is an inhibitor of efflux pumps (Kolaczkowski *et al*., 2003), and an MIC of 98 μg ml$^{-1}$ was obtained for all tested isolates. After MIC determination, each isolate was tested against fluconazole and itraconazole combined with promethazine at 20 μg ml$^{-1}$. MICs for fluconazole dropped from $> 64$ to 2 μg ml$^{-1}$ for both *C. albicans* strains and from $> 64$ to 16 μg ml$^{-1}$ for the *C. tropicalis* isolate. As for itraconazole, MICs declined from $> 16$ to 0.0625 μg ml$^{-1}$ for all three strains. These data suggest that the antifungal resistance observed for these *Candida* isolates was possibly associated with the overexpression of efflux pumps, since pump inhibition resulted in the decrease of azole MICs.

As for phospholipase production, out of the 23 tested *Candida* isolates, eight produced phospholipase (6 *C. albicans*, 1 *C. famata* and 1 *C. parapsilosis*). Although the sampled raptors were apparently healthy, all six positive *C. albicans* isolates presented strong enzymatic activity, with PZ values ranging from 0.36 to 0.55, similar to what was observed by Sidrim and colleagues 2010, who also isolated a high percentage of strongly positive phospholipase producing *C. albicans*. These findings corroborate those of Cafarchia and colleagues (2006b), who stated that raptors are carriers of potentially pathogenic and zoonotic yeast species, once elevated phospholipase production may be associated with enhanced virulence (Ibrahim *et al*., 1995; Ghannoum, 2000).

Another noteworthy finding of this study was the recovery of three resistant *Candida* isolates from the crop of two *R. magnirostris* (1 *C. albicans* and 1 *C. tropicalis*) and the cloaca of one *C. atratus* (1 *C. albicans*). These three birds were free-ranging individuals from the city of Fortaleza that had recently been taken into captivity to recover from small traumas. Thus, it is known that they had never been subjected to antifungal therapy. These species are very common in urban centres, contributing to their exposure to various chemical compounds, including those from improper waste management. From 1978 to 1998, the municipal solid waste of the city of Fortaleza was destined to an open-air garbage dump, located near the Cocó River Basin. Currently, although officially deactivated, the site still receives solid waste and still contributes to environmental pollution, especially because the residues and the area do not receive proper treatment (Santos and Rigotto, 2008).

In addition, the occurrence of cross-resistance to medical and agricultural azoles in *Candida* spp. (Müller *et al*., 2007) may also have contributed to these findings, once the two *R. magnirostris* individuals may have been exposed to azoles used in agriculture through the ingestion of small herbivore preys. However, this possibility seems less likely because Fortaleza is a big city where agriculture is not a common practice. Besides, the American black vulture (*C.atratus*) is a scavenger species (Carvalho *et al*., 2003), thus, prey ingestion would not play an important role for the recovery of resistant yeasts.

Azole resistance has also been observed in environmental isolates of *Aspergillus* spp. (Mortensen *et al*., 2010) and these resistant strains are also capable of infecting humans, resulting in azole-resistant cases of aspergillosis (Snelders *et al*., 2009). Similarly, these findings concerning azole resistance in *Candida* strains from raptors also raise an important public health issue, considering that animals can represent a source of *Candida* spp. infections for humans (Edelmann *et al*., 2005).

Historically, raptors have been used as sentinels for the presence of environmental contaminants, such as pesti-

cides and heavy metals (Marrow *et al*., 2009). Because of their predatory behaviour, once microorganisms from the gut of preys colonize the gastrointestinal tract of raptors, the use of these birds as indicators of the presence of resistant bacteria has also been encouraged, since their microbiota reflects the environment they inhabit (Marrow *et al*., 2009; Guenther *et al*., 2010).

Considering the high rate of yeast isolation from animal sources, especially *Candida* spp. (Melville *et al*., 2004; Vieira and Acqua-Coutinho, 2009; Brilhante *et al*., 2010; 2011), these microorganisms could also provide valuable information on animals' habitats. Based on this, *Candida* spp. could be used as indicators of environmental pollution, through phenotypical assessment of their *in vitro* susceptibility profile, since azole resistance is frequently associated with the overexpression of efflux pumps (Feng *et al*., 2010), which is possibly related to the exposure of these microorganisms to chemical compounds (e.g. pollutants), as an unspecific mechanism of cellular detoxification (Jungwirth and Kuchler, 2006). Additionally, the use of raptors for this purpose seems particularly interesting because free-ranging individuals are commonly taken to rehabilitation centres where they can easily and non-invasively be assessed, contributing to environmental monitoring.

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