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Evidence of Fluconazole-Resistant *Candida* Species in Tortoises and Sea Turtles

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Abstract The aim of this study was to evaluate the antifungal susceptibility of *Candida* spp. recovered from tortoises (*Chelonoidis* spp.) and sea turtles (*Chelonia mydas*, *Caretta caretta*, *Lepidochelys olivacea*, *Eretmochelys imbricata*). For this purpose, material from the oral cavity and cloaca of 77 animals (60 tortoises and 17 sea turtles) was collected. The collected specimens were seeded on 2 % Sabouraud dextrose agar with chloramphenicol, and the identification was carried out by morphological and biochemical methods. Sixty-six isolates were recovered from tortoises, out of which 27 were *C. tropicalis*, 27 *C. famata*, 7 *C. albicans*, 4 *C. guilliermondii* and 1 *C. intermedia*, whereas 12 strains were obtained from sea turtles, which were identified as *Candida parapsilosis* ($n = 4$), *Candida guilliermondii* ($n = 4$), *Candida tropicalis* ($n = 2$), *Candida albicans* ($n = 1$) and *Candida intermedia* ($n = 1$). The minimum inhibitory concentrations for amphotericin B, itraconazole and fluconazole ranged from 0.03125 to 0.5, 0.03125 to >16 and 0.125 to >64, respectively. Overall, 19 azole-resistant strains (14 *C. tropicalis* and 5 *C. albicans*) were found. Thus, this study shows that Testudines carry azole-resistant *Candida* spp.

Keywords *Candida* spp. · Testudines · Antifungal susceptibility · Resistance

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Introduction

The popularity of keeping reptiles in captivity is growing and has raised concerns about the risks to human health due to the lack of information on the microbiota of these animals. Reptiles can represent a potential source of infection for humans, since they harbor potentially pathogenic microorganisms [1], including yeasts of the genera *Candida*, *Rhodotorula* and *Trichosporon* [2]. These animals can act as carriers and disseminators of potentially pathogenic yeasts and contribute to the infection in humans and other animals [3].

The incidence of human infections caused by *Candida* spp. has increased, especially in immunocompromised individuals and in patients with cancer [4, 5]. *Candida albicans* is the most frequently found species, but non-*albicans Candida*, such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. famata*, have emerged as important pathogens. Moreover, many of these strains from patients with candidemia are resistant to azoles, especially in previously treated individuals. Recent studies have shown high rates of azole resistance in *Candida* spp. from animals, such as dogs [6], birds [7, 8], prawns [9] and horses [10]. However, there are no reports on the antifungal susceptibility of *Candida* spp. from reptiles.

Thus, considering that animals can act as carriers of resistant *Candida* strains, contributing to the maintenance and dissemination of these yeasts in the environment, and among humans and other animals, the aim of this study was to evaluate the antifungal susceptibility of *Candida* spp. recovered from tortoises (*Chelonoidis* spp.) and sea turtles (*Chelonia mydas*, *Caretta caretta*, *Lepidochelys olivacea*, *Eretmochelys imbricata*).

Materials and Methods

A total of 77 animals were assessed in this study: 60 tortoises (*Chelonoidis* spp.) kept in captivity at the Rehabilitation Center for Wildlife (CETAS) of the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA, 3°49′50.4948″S, 38°28′40.0362″W) ($n = 26$), Ecopoint Zoo (3°45′55.0296″S, 38°34′34.6512″W) ($n = 21$), TAMAR Project (2°56′18″S, 39°48′51″W) ($n = 8$) and Sargento Prata Zoo (3°48′36.3162″S, 38°32′4.365″W) ($n = 5$); and 17 sea turtles found stranded alive on beaches in Ceará state, which were taken for rehabilitation at CETAS (5 *C. mydas*, 1 *L. olivacea*, 1 *E. imbricata* and 1 *C. caretta*) and TAMAR Project (5 *C. mydas*, 4 *L. olivacea*). All assessed animals were subjected to physical examination, including integument, oral and ocular mucosae inspection for the presence of lesions and ectoparasites and for the evaluation of the hydration status, and assessment of the muscle tone and responsiveness to handling. All tortoises were clinically healthy at the time of sampling, sea turtles that were sampled at TAMAR Project were clinically healthy because they had already been rehabilitated, while those sampled at the Rehabilitation Center for Wildlife were clinically ill, as they had just been rescued, mainly presenting gastrointestinal disorders, due to the accidental ingestion of foreign bodies associated with sea water pollution, or respiratory disorders, with signs of uneven floating. This study was approved by the Ethics Committee on Animal Research of the Federal University of Ceará (number 02/2013) and by SISBIO (number 36357-1) of the Chico Mendes Biodiversity Conservation Institute.

Sample Collection and Yeast Identification

Samples were collected from the oropharynx and cloaca as described in Brilhante et al. [11]. Briefly, the swabs were inserted into the anatomical site and rotated, and then placed into sterile glass tubes with sterile saline (0.9 % NaCl) at 4 °C until processing. The swabs were seeded on 2 % Sabouraud dextrose agar (SDA, Difco Laboratories) with chloramphenicol and incubated at 25 °C for 10 days. Colonies suggestive of *Candida* were chosen based on their morphological features, including texture and color, and confirmed through microscopic evaluation. Each morphological type was subjected to the phenotypical identification procedures. When several colonies with the same morphological features were recovered, they were randomly sampled (up to ten colonies), inoculated in 5 mL of saline solution and seeded on chromogenic medium (Candida HiChrome Differential Agar, HiMedia, India) for the identification of mixed *Candida* species. The isolated strains were grown on Corn meal-Tween-80 agar for micromorphological analysis. Then, they were also assessed for their ability of fermenting and assimilating carbohydrates [12]. In cases of dubious identification, Vitek 2 (bioMérieux, France) was used for yeast identification [11].

Antifungal Susceptibility Testing

Candida spp. were tested against amphotericin B (AMB, Sigma, USA), fluconazole (FLC, Pfizer, Brazil) and itraconazole (ITC, Janssen Pharmaceutica, Belgium), according to the document M27-A3 [13]. The test was performed in 96-well microdilution plates and incubated at 35 °C for 48 h. For AMB, the minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no growth was

Table 1 Distribution of *Candida* spp. in the gastrointestinal tract of tortoises ($n = 60$) and sea turtles (17)

Species	Tortoise		Sea turtle		Total
	Oral	Cloaca	Oral	Cloaca	
C. tropicalis	12	15	2	–	29
C. famata	13	14	–	–	27
C. albicans	3	4	1	–	8
C. guilliermondii	3	1	1	3	8
C. parapsilosis complex	–	–	4	–	4
C. intermedia	–	1	1	–	2
Total	31	35	9	3	78

observed. For ITC and FLC, the MICs were defined as the lowest drug concentration able to inhibit 50 % of fungal growth, when compared to the control [13]. Isolates with MIC > 1 and ≥ 1 μg/mL were considered resistant to AMB and ITC, respectively [13]. *C. albicans*, *C. parapsilosis* and *C. tropicalis* were considered resistant to FLC when MIC ≥ 8 μg/mL [14]. *C. parapsilosis* ATCC 22019 was included as quality control for each test [13].

Statistical Analysis

Fisher's exact test was used for comparison of positivity for the different *Candida* species, between species of animals and anatomical sites. ANOVA was performed to test the difference in MICs between the different *Candida* species, and the Student's *t* test for independent samples to compare the MICs between the animal species. In all cases, the maximum level of significance adopted for affirmative conclusions was 5 %.

Results

Overall, 40/60 tortoises were positive for the presence of *Candida* sp. This yeast genus was recovered from the oral cavity of 8/40 (20 %), the cloaca of 13/40 (32.5 %) and both sites of 19/40 (47.5 %) individuals. As for sea turtles, 5/17 animals were positive for *Candida*, of which 3/5 (60 %) presented this yeast genus only in the oral cavity, while 2/5 (40 %) presented it in both anatomical sites. An average of 20–40 yeast colonies were observed in each positive agar plate.

Seventy-eight *Candida* strains from tortoises (66/78, 84.6 %) and sea turtles (12/78, 15.4 %) were isolated. *C. mydas* was the only sea turtle species from which *Candida* spp. were recovered. Six species of *Candida* were identified, of which the most frequently isolated were *C. tropicalis* ($n = 29$) and *C. famata* ($n = 27$), followed by *C. albicans* ($n = 8$), *C. guilliermondii* ($n = 8$), *C. parapsilosis* species complex ($n = 4$) and *C. intermedia* ($n = 2$; Table 1).

C. parapsilosis was not recovered from tortoises; thus, it was statistically more prevalent in *C. mydas* ($P = 0.02$), when compared to the former. *C. tropicalis* was more prevalent in the cloaca of tortoises than sea turtles ($P = 0.03$), while *C. guilliermondii* was more prevalent in the cloaca of sea turtles than tortoises ($P = 0.05$).

The results obtained from the antifungal susceptibility tests are shown in Table 2. No resistance to amphotericin B was observed among the isolates. Only one strain from sea turtle (*C. tropicalis*) was resistant to itraconazole and fluconazole, while, among those strains from tortoises, 13 *C. tropicalis* were azole-resistant, nine to fluconazole and itraconazole and four only to fluconazole. As for *C. albicans* from tortoises, five strains were resistant, one to itraconazole and four to fluconazole. Amphotericin B ($P = 0.0056$) and itraconazole ($P = 0.0310$) MICs against *C. tropicalis* from *Chelonoidis* spp. were higher than those obtained against strains from *C. mydas*.

Discussion

The present study focused on the isolation of *Candida* spp. from Testudines (tortoises and turtles). There are reports of gastrointestinal [15] and pulmonary candidiasis [16] in tortoises; however, the impact of yeasts from sea turtles on human and animal health is still unknown. In this study, the recovery rate of *Candida*

Table 2 In vitro antifungal susceptibility of *Candida* species isolated from tortoises and sea turtles

Candida spp. (*n*)	MIC (μg/ml)		
	AMB	ITR	FLU
C. tropicalis (29)	0.03125 (1)	0.03125 (11)	0.125 (2)
	0.0625 (7)	0.0625 (3)	0.25 (2)
	0.125 (8)	0.125 (2)	0.5 (2)
	0.25 (13)	0.25 (3)	2 (5)
		0.5 (1)	4 (5)
		2 (2)[a]	8 (2)[a]
		>16 (7)[a]	16 (3)[a]
			32 (3)[a]
			64 (2)[a]
			>64 (3)[a]
C. famata (27)	0.03125 (6)	0.03125 (14)	0.25 (4)
	0.0625 (9)	0.0625 (5)	0.5 (4)
	0.125 (6)	0.125 (6)	1 (6)
	0.25 (5)	0.25 (2)	2 (1)
	0.5 (1)		4 (6)
			8 (2)
			16 (1)
			32 (3)
C. albicans (8)	0.03125 (1)	0.03125 (1)	0.125 (1)
	0.0625 (1)	0.0625 (3)	0.25 (1)
	0.125 (3)	0.125 (2)	1 (1)
	0.25 (3)	0.5 (1)	4 (1)
		1 (1)[a]	16 (2)[a]
			32 (2)[a]
C. guilliermondii (8)	0.03125 (4)	0.03125 (5)	0.125 (2)
	0.0625 (1)	0.0625 (2)	0.5 (2)
	0.25 (2)	0.125 (1)	1 (1)
	0.5 (1)		2 (2)
			4 (1)
C. parapsilosis (4)	0.03125 (1)	0.03125 (1)	0.5 (2)
	0.25 (1)	0.0625 (2)	1 (1)
	0.5 (2)	0.125 (1)	2 (1)
C. intermedia (2)	0.0625 (2)	0.0625 (2)	1 (2)

AMB amphotericin B, *FLC* fluconazole, *ITC* itraconazole

[a] Antifungal resistance (ITC ≥ 1 and FLU ≥ 8 μg/mL)

spp. from tortoises (66 %) was greater than that observed for sea turtles (29.4 %). The tortoises assessed in this study were kept in outdoor group enclosures with soil substrate, with close contact with each other, and were fed with a diversity of fruits and vegetables, which were offered on the ground, on a concrete surface. Considering the high number of individuals within the enclosures and their habits of eating and sleeping together, these animals most likely share several commensal microorganisms. In contrast, sea turtles are solitary open water animals. When in captivity, these animals are commonly kept in water tanks, individually or in small groups, and are fed with algae, fish, crustacean and mollusk, depending on the turtle species. Interestingly, *Candida* spp. were only isolated from *C. mydas* under critical rehabilitation at CETAS, where several other animals, including birds, mammals and other reptiles, were also kept. This finding suggests that *Candida* spp. are not usual commensal microorganisms in the oral cavity and

cloaca of healthy sea turtles. Indeed, the presence of *Candida* in these animals was most likely associated with their impaired immunity or possibly with the promiscuity of the rehabilitation facilities.

Considering the opportunistic features of *Candida* spp., veterinarians and biologists should be aware of changes that can affect the host–parasite balance, such as stress, poor hygiene, overcrowding and poor nutrition, since they can favor infections with these yeasts and facilitate dissemination among confined animals [17, 18]. Veterinarians and caretakers must establish good management practices to prevent or reduce the predisposing conditions for the occurrence of infections caused by opportunistic pathogens. Moreover, these professionals should be aware that these animals may act as sources of infection for humans and other animals, especially with *Salmonella* spp. [2]. Hence, certain sanitary measures must be adopted, including thorough hand washing after handling these animals, dedicating kitchen and medical utensils for reptiles in zoos, rehabilitation facilities and households, cleaning the enclosures and utensils with sodium hypochloride and separating an area away from food preparation areas for disposing reptile wastewater and droppings and washing used materials [19].

C. tropicalis and *C. famata* were the most frequently found species in tortoises. The high frequency of isolation of *C. tropicalis* corroborates the findings of Benites et al. [2], who detected a high percentage (22 %) of this species. However, these authors did not recover *C. famata*, which was one of the most isolated species in the present study. Other authors have shown that *C. tropicalis* is prevalent in soils enriched with organic matter and aquatic environments and in synanthropic wild birds [20–22]. In addition, this species is one of the most prevalent in cancer or postoperative patients in intensive care units, associated with high lethality rates [23]. *C. famata* has been widely isolated from marine waters, polluted aquatic environments and fish [24] and has been responsible for candidemia and ocular and central nervous system infections. Among the isolates from sea turtles, *C. parapsilosis* and *C. guilliermondii* were the most frequent.

Regarding the in vitro antifungal susceptibility, we did not observe resistance to amphotericin B, showing that the strains isolated from tortoises and turtles exhibit susceptibility to this drug similar to that observed for *Candida* spp. recovered from other healthy animals [6, 8–11]. Resistance to azoles was observed in *C. tropicalis* and *C. albicans* strains, corroborating previous observation of resistance in *Candida* spp. isolated from animals and environmental sources [7, 8, 21, 24]. Resistance to azoles can be associated with mutations occurring in gene sequence or expression of these genes, arising due to selective pressures induced by the use of azoles [25]. Since the animals evaluated in this study had no history of prior treatment with antifungal drugs, the resistance can be associated with the presence of these compounds in the diet, especially fruits and vegetables given to captive animals, since azole derivatives are also used in agriculture.

Finally, the data show that Testudines carry antifungal-resistant *C. tropicalis* and *C. albicans*, emphasizing the importance of monitoring these animals, since they can contribute to the environmental dissemination of these microorganisms and act as potential sources of human infections.

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Compliance with Ethical Standards

Conflict of interest None to declare.

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