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Original Contribution

Antifungal Resistance and Virulence Among *Candida* spp. from Captive *Amazonian manatees* and West Indian Manatees: Potential Impacts on Animal and Environmental Health

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Abstract: This work aimed at evaluating the antifungal susceptibility and production of virulence factors by *Candida* spp. isolated from sirenians in Brazil. The isolates (n = 105) were recovered from the natural cavities of Amazonian and West Indian manatees and were tested for the susceptibility to amphotericin B, itraconazole, and fluconazole and for the production of phospholipases, proteases, and biofilm. The minimum inhibitory concentrations (MICs) for amphotericin B ranged from 0.03 to 1 μg/mL, and no resistant isolates were detected. Itraconazole and fluconazole MICs ranged from 0.03 to 16 μg/mL and from 0.125 to 64 μg/mL, respectively, and 35.2% (37/105) of the isolates were resistant to at least one of these azole drugs. Concerning the production of virulence factors, phospholipase activity was observed in 67.6% (71/105) of the isolates, while protease activity and biofilm production were detected in 50.5% (53/105) and 32.4% (34/105) of the isolates, respectively. Since the natural cavities of manatees are colonized by resistant and virulent strains of *Candida* spp., these animals can act as sources of resistance and virulence genes for the environment, conspecifics and other animal species, demonstrating the potential environmental impacts associated with their release back into their natural habitat.

Keywords: yeasts, azole resistance, virulence factors, sirenians

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Introduction

Antimicrobial resistance is a global public health problem in human and veterinary medicine and it has also been detected in microorganisms isolated from wildlife (Radhouani et al. 2014). Even though these animals are not frequently treated with antimicrobials, resistant microorganisms have been recovered from them, most likely as a result of their contact with humans, domestic animals and aquatic and terrestrial environments contaminated with effluents and wastewater (Radhouani et al. 2014; Carroll et al. 2015). In addition, wild animals, particularly migratory species, may carry resistant microorganisms, hence, contributing to the dissemination of bacterial and fungal resistance genes (Radhouani et al. 2014).

Resistance to multiple antibiotics has been detected in bacteria recovered from aquatic mammals, including captive, stranded and/or free-ranging cetaceans, sirenians, pinnipeds, and mustelids (Rose et al. 2009; Schaefer et al. 2009; Brownstein et al. 2011; Hower et al. 2013; Potter 2013; Wallace et al. 2013; Attademo 2014; Berardi et al. 2014). However, systematic microbiological studies on these animals are still scarce, when compared to data on terrestrial animals (Rose et al. 2009).

Concerning the detection of antifungal resistance in yeasts from aquatic mammals, only few studies have been carried out. Takahashi et al. (2010), for example, detected resistance to itraconazole and fluconazole in *Candida albicans* and *C. tropicalis* from the respiratory tract of captive bottlenose dolphins, and Cordeiro et al. (2014) found azole resistance in one *C. tropicalis* strain recovered from a dwarf sperm whale that stranded on the northeast coast of Brazil.

Despite the growing importance of microorganisms as agents of infectious diseases in aquatic mammals, as well as their use as indicators of the health of aquatic ecosystems (Bossart 2010), studies on the virulence features of bacteria and fungi from these animals are still scarce. Hughes et al. (2013), for example, reported the presence of virulence genes in *Vibrio* strains from harbor seals in California, while Cordeiro et al. (2014) have assessed the production of virulence factors by *C. tropicalis* from veterinary sources, including cetaceans stranded in Brazil.

The Order Sirenia comprises four living species, three manatees and the dugong, which are all classified as endangered species, mainly due to high mortality rates associated with human activities (Marsh et al. 2011; IUCN 2013). Knowledge on the health status of sirenians is still scarce for many populations around the world and the role of microorganisms as components of the microbiota and/or agents of infectious diseases remains unknown. However, this information is essential for an accurate assessment of population health, contributing to the conservation of these species (Bonde and Aguirre 2004; Marsh et al. 2011).

There are no published data on the antifungal susceptibility and virulence of fungi recovered from sirenians, even though this information is relevant for preventing and treating fungal infections and for using these animals as sentinels for environmental health. Hence, this research aimed at evaluating the antifungal susceptibility and production of virulence factors by *Candida* spp. isolated from Amazonian manatees and West Indian manatees kept in captivity in Brazil for rehabilitation and eventual release into the wild.

Material and Methods

Ethical Aspects

This study was authorized by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), license SISBIO No. 35950-1, and by the Animal Research Ethics Committee of State University of Ceará, under the number 12639479-2.

Animals

The *Candida* isolates analyzed in this study were recovered from the natural cavities (oral cavity, nostrils, genital opening, and rectum), of 50 Amazonian manatees (*T. inunguis*) and 26 West Indian manatees (*T. manatus*). Biological samples for yeast recovery were collected from each anatomical site, through the insertion of sterile cotton swabs (Sidrim et al. 2015). The assessed animals are held in captivity at three Brazilian institutions that have rescue, rehabilitation and release programs with these species: Centro de Preservação e Pesquisa de Mamíferos Aquáticos (CPPMA)/Eletrobras Amazonas Energia, in the State of Amazonas, where the Amazonian manatees were maintained, and Centro Mamíferos Aquáticos (CMA/ICMBio), in the State of Pernambuco, and Associação de Pesquisa e

Preservação de Ecossistemas Aquáticos (AQUASIS), in the State of Ceará, where the West Indian manatees were maintained. These animals have been in captivity for different periods of time: 0–5 years ($n = 29$), >5–10 years ($n = 16$) and >10 years ($n = 31$). All animals were assessed in the period from October 2012 to November 2013.

The manatees belonged to both sexes (11 male and 15 female *T. inunguis* and 24 male and 26 female *T. manatus*) and different age groups (6 calves, 15 juveniles and 29 adult *T. inunguis* and 5 newborns, 2 calves, 11 juveniles and 8 adult *T. manatus*). The animals received different diets (artificial milk formulas or vegetables), according to age and internal institutional protocols. All animals received a single clinical score (poor, medium, good, or excellent) considering the nutritional status, presence of external lesions, clinical history and use of antimicrobials. Regarding *T. inunguis*, 88% (44/50) of the animals received excellent score, 8% (4/50) good and 4% (2/50) medium score, while 69.3% (18/26) *T. manatus* received excellent clinical score, 11.5% (3/26) good, 11.5% (3/26) medium, and 7.7% (2/26) poor (Sidrim et al. 2015). Conditions for maintenance in captivity also varied, such as water treatment (chlorinated or untreated), salinity (salt or fresh) and density of animals per tank. *T. inunguis* specimens were kept in eight different tanks, as follows: three calves and one adult in individual tanks, two calves in one tank and three collective tanks containing 16, 16, and 12 adult/juvenile animals. *T. manatus*, on the other hand, were kept in nine different tanks: two calves, one juvenile and one adult in individual tanks, one collective tank containing five newborn calves and four collective tanks containing 3, 4, 4, and 6 adult/juvenile animals.

Yeasts

For this research, 105 *Candida* isolates recovered from natural cavities of Amazonian manatees (*Trichechus inunguis*) and West Indian manatees (*T. manatus*) were used. These isolates were recovered on Petri dishes containing Sabouraud dextrose 2% agar plus chloramphenicol (0.05 g/L), and identified through phenotypical characteristics, such as macromorphological and micromorphological analyses and biochemical tests, including urease production, carbohydrate and nitrogen assimilation and carbohydrate fermentation. The identification of cryptic specie (*Candida albicans-Candida dubliniensis* and *Candida parapsilosis* species Complex) was confirmed through molecular analyses (Sidrim et al. 2015). The recovered *Candida* isolates are kept in the culture collection of the Specialized Medical Mycology Center (CEMM) and belong to the following species: *C. albicans* (52/105), *C. parapsilosis sensu stricto* (14/105), *Candida orthopsilosis* (4/105), *Candida metapsilosis* (3/105), *Candida guilliermondii* (12/105), *Candida pelliculosa* (3/105), *Candida tropicalis* (6/105), *Candida glabrata* (2/105), *Candida famata* (5/105), *Candida ciferri* (1/105), *Candida norvegensis* (1/105), and *Candida krusei* (2/105).

Antifungal Susceptibility Testing

The tested *Candida* spp. isolates ($n = 105$) were submitted to antifungal susceptibility tests through broth microdilution, in 96-well polystyrene plates, using RPMI 1640 broth, as the growth medium, according to the recommendations of the document M27-A3 of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2012). Inocula were prepared with 24-h-old cultures, adjusted to 0.5 on McFarland scale, then, diluted with RPMI 1640 until reaching a final concentration of 0.5 to 2.5×10^3 cfu/mL. The antifungal drugs were tested at concentrations ranging from 0.03125 to 16 mg/mL for amphotericin B (AMB) and itraconazole (ITC) and from 0.125 to 64 mg/mL for fluconazole (FLC). Plates were incubated at 35°C for 48 h, followed by visual readings. The minimum inhibitory concentration (MIC) for AMB was defined as the lowest concentration capable of inhibiting 100% of fungal growth, while the MICs for ITC and FLC were defined as the lowest concentration capable of inhibiting 50% of fungal growth, when compared to growth control. Overall, MICs >1, ≥1 and ≥64 μg/mL indicated resistance to AMB, ITC and FLC, respectively. Concerning *C. albicans*, *C. parapsilosis* complex and *C. tropicalis*, MICs ≥8 μg/mL, indicated *in vitro* resistance to FLC. As quality control, the strain *C. parapsilosis* ATCC 22019 was included in the tests (CLSI 2012).

Production of Virulence Factors

Phospholipases

The strains of *Candida* spp. ($n = 105$) were grown on potato dextrose agar, incubated at 35°C for 48 h. Then, fungal inocula were prepared and adjusted to a turbidity of 4 on McFarland scale. Afterwards, 5 μL of each fungal inoculum were transferred to on 5-mm sterile filter paper disks, which were then placed on Petri dishes containing egg yolk

agar. The egg yolk agar consisted of 2% Sabouraud dextrose agar, supplemented with 1 mol/L of sodium chloride, 0.05 mol/L of calcium chloride and 8% of sterile egg yolk emulsion at 30%. The Petri dishes were incubated at 35°C for seven days. Phospholipase activity (Phz) was determined by calculating the ratio between the diameter of the colony and the total diameter (colony + precipitation zone). When Phz = 1, the isolates were negative for phospholipase production, when $0.64 \leq Phz < 1$ the isolates were positive for enzymatic production and when $Phz < 0.64$, the isolates were strongly positive for phospholipase activity (Sidrim et al. 2010; Brilhante et al. 2011).

Proteases

The strains of *Candida* spp. ($n = 105$) were grown in YEPD broth (1% yeast extract, 2% peptone, 2% dextrose) and incubated at 35°C, for 24 h. Then, inocula were prepared with turbidity corresponding to 5 on McFarland scale. Afterwards, 10 µL of each fungal inoculum were disposed on 5-mm sterile filter paper disks, which were then placed on Petri dishes containing bovine serum albumin agar. This agar was prepared with 2% dextrose, 0.1% yeast extract, 0.5% NaCl, 0.25% K2HPO4, 0.02% MgSO4 7H2O, and 1.5% agar, supplemented with a 0.25% solution of bovine serum albumin, after cooling to 50°C. The plates were incubated at 37°C, for 5 days. The proteolytic activity (Pz) was determined by calculating the ratio between the diameter of the colony and the total diameter (colony + proteolysis zone). Pz values of 1 indicated the absence of proteolytic activity, while Pz <1 indicated protease production (Vidotto et al. 2004).

Biofilm

The strains of *Candida* spp. ($n = 105$) were analyzed for their biofilm-forming ability, according to Peeters et al. (2008) and Ravi et al. (2009), with modifications. Initially, the isolates were grown on Sabouraud agar at 30°C, for 48 h. Then, they were transferred to Sabouraud broth and incubated under agitation (150 rpm), for 24 h. Afterwards, the tubes were centrifuged (3000 rpm, 10 min), the supernatant was discarded, and the pellet was washed twice with sterile PBS. The supernatant was removed and the pellet was resuspended in RPMI 1640 medium, adjusting the inoculum to 0.5 to 1 on the McFarland scale. Afterwards, 100 µL of the resulting inoculum were placed in 96-well flat-bottomed polystyrene microtiter plates. Each fungal isolate was tested in triplicate and the plates were incubated, under agitation (150 rpm), at 37°C, for 24 h. Three wells were used for positive and negative growth control. After incubation, the supernatant was carefully aspirated from the wells, followed by three washes with PBS-Tween. Then, the wells were washed with 100 µl of 100% methanol. After drying, 100 µL of a 0.3% crystal violet solution were added. After 20 min, the crystal violet was removed, and the wells were washed twice with 200 µL of sterile distilled water. Then, 150 µL of 33% acetic acid solution was added to stained wells and left for 30 s. The acetic acid was, then, transferred to another 96-well polystyrene plate, which was read through spectrophotometry at a wavelength of 540 nm. The optical density values obtained at 540 nm (OD540 nm) were corrected by subtracting the OD540 nm obtained for the negative control (containing only RPMI 1640 broth). The cutoff point (ODc) for biofilm production was defined as three standard deviations above the mean OD540 nm obtained for the negative control. At the end, all the tested strains were classified according to Stepanovic et al. (2000), as non-biofilm producers ($OD540\ nm \leq ODc$), weak producers ($ODc < OD540\ nm \leq 2xODc$), moderate producers ($2xODc < OD540\ nm \leq 4xODc$) and strong producers ($4xODc < OD540\ nm$).

Statistical Analysis

In order to verify the differences in antifungal drug MICs between different variables, the MIC values were submitted to Log2 transformation. Based on this transformation, the data were analyzed through linear regression models, using the Log2 MIC values as the explanatory variable. Phospholipase, protease, and biofilm production and the categorical data concerning host species, host age and collection site were used as the dependent variables, by applying dummy variables. Analyses of variance and post hoc Tukey's test were used to assess differences in phospholipase, protease, and biofilm production between the different *Candida* species. *P* values lower or equal to 5% indicated statistically significant results.

Results

Antifungal Susceptibility Testing

Amphotericin B MICs against the tested isolates of *Candida* spp. ($n = 105$) ranged from 0.03 to 1 mg/mL and none of

the isolates were resistant to this drug. The amphotericin B MICs against *C. albicans* were statistically higher than those obtained against the other *Candida* species ($P = 0.02$).

Regarding the azoles, the MICs ranged from 0.03 to 16 μg/mL for itraconazole and from 0.125 to 64 μg/mL for fluconazole. Among these isolates, 35.2% (37/105) were resistant to at least one of the azoles, with similar prevalence rate among *T. inunguis* and *T. manatus*. Resistant strains were obtained from all anatomical sites evaluated.

Among the resistant isolates, 86.49% (32/37) were *C. albicans*, 10.81% (4/37) *C. tropicalis,* and 2.70% (1/37) *C. guilliermondii*. Considering these isolates, 48.65% (18/37) were resistant to both azoles, 40.54% (15/37) only to itraconazole and 10.81% (4/37) to fluconazole. High resistance rates were detected in *C. albicans* and *C. tropicalis*, reaching proportions of 61.54% (32/52) and 66.67% (4/6), respectively. Itraconazole MICs against *C. albicans* were statistically higher than those obtained against the other *Candida* species ($P = 0.01$). The MIC values obtained for all isolates of *Candida* spp. from both hosts are described in Table 1.

Production of Virulence Factors

Phospholipases

Among the evaluated isolates of *Candida* spp., 52.38% (55/105) were positive ($0.64 \leq Phz < 1$), 15.24% (16/105) were strongly positive ($Phz < 0.64$) and 32.38% (34/105) were negative ($Phz = 1$) for the production of phospholipases, with a similar profile between *Candida* spp. from both host species (Table 2).

The species that presented higher phospholipase production were *C. albicans* (51/52), *C. glabrata* (2/2), *C. krusei* (2/2), *C. pelliculosa* (3/3), and *C. tropicalis* (4/6). *C. albicans* presented statistically higher phospholipase production ($P < 0.05$), when compared to the other *Candida* spp. A positive correlation was observed between amphotericin B MICs and phospholipase production ($P = 0.01$).

Proteases

Among the evaluated strains of *Candida* spp., 50.48% (53/105) were positive ($Pz < 1$) and 49.52% (52/105) were negative ($Pz = 1$) for the production of proteases, with a similar profile between *Candida* spp. from both host species. *C. albicans* presented statistically higher protease production ($P < 0.05$), when compared to the other *Candida* spp., reaching up to 88.46% (46/52) of positivity (Table 3). A positive correlation was observed between amphotericin B ($P = 0.03$) and itraconazole ($P = 0.04$) MICs and protease production.

Biofilm

Of the 105 tested *Candida* isolates, 67.62% (71/105) were classified as non-biofilm producers, 21.90% (23/105) as low producers, 6.67% (7/105) as moderate producers and 3.81% (4/105) as strong biofilm producers (Table 4). The species of *Candida* that presented the greatest percentages of biofilm-producing isolates were *C. tropicalis* with 66.67% (4/6), *C. orthopsilosis* with 50% (2/4), *C. krusei* with 50% (1/2), and *C. parapsilosis sensu stricto* with 50% (7/14). Among the antifungal resistant strains, 31.25% (10/32) of *C. albicans* and 75% (3/4) of *C. tropicalis* were positive for biofilm production, but no correlations were observed between these two aspects. In particular, the resistant isolates of *C. albicans* from West Indian manatees had higher percentages of biofilm-producing strains (83.34%; 5/6), when compared to those from Amazonian manatees (19.23%; 5/26).

Discussion

This study presents the first data on the antifungal susceptibility profile of *Candida* spp. isolated from sirenians. Among the three azole resistant *Candida* species, high resistance rates were found in *C. albicans* and *C. tropicalis*, corroborating previous studies with other species of domestic and wild animals (Brito et al. 2009; Sidrim et al. 2010; Brilhante et al. 2011; Brilhante et al. 2012; Brilhante et al. 2013). Knowledge on the origin of antimicrobial resistance in wildlife is important for human health due to the increasing impact of zoonoses on public health and the need to predict the emergence of resistant pathogens (Radhouani et al. 2014). The development of antifungal resistance can be multifactorial. Antifungal drugs are rarely used in a systematic way to treat sirenians, except for healing ointments with multiple compounds that are commonly applied on wounds and abscesses (Bossart 2001; D'Affonseca Neto and Vergara-Parente 2007). Additionally, the use of some antibacterials, such as metronidazole, could also lead to resistance in *Candida* spp. due to a low grade antifungal effect (Ben-Ami et al. 2012; Arendrup 2013).

It is known that close contact of wild animals with human beings favors the transmission of resistant

Table 1. Antifungal Susceptibility of *Candida* spp Isolated from Sirenians.

Host	Site	Yeast species	MIC range			Resistant isolates	
			AMB	ITC	FLC	ITC	FLC
Trichechus inunguis	Oral	*C. albicans* (*n* = 26)	0.25–1	0.03–16	0.125–64	13	6
		C. parapsilosis sensu stricto (*n* = 3)	0.06–0.25	0.03–0.06	0.5–2	–	–
		C. orthopsilosis (*n* = 1)	0.25	<0.03	0.5	–	–
		C. guilliermondii (*n* = 2)	0.25	0.06–0.25	1–8	–	–
	Nasal	*C. glabrata* (*n* = 1)	0.25	0.125	4	–	–
		C. parapsilosis sensu stricto (*n* = 2)	0.125–0.25	0.03	1–2	–	–
		C. metapsilosis (*n* = 1)	0.25	0.03	4	–	–
		C. ciferri (*n* = 1)	0.25	0.125	4	–	–
	Genital	*C. albicans* (*n* = 11)	0.25–1	0.03–16	0.125–32	7	5
		C. guilliermondii (*n* = 3)	0.125–1	0.125–0.25	2–8	–	–
		C. krusei (*n* = 1)	1	0.125	16	–	–
		C. norvegensis (*n* = 1)	0.03	0.06	1	–	–
		C. parapsilosis sensu stricto (*n* = 4)	0.06–0.25	0.03	1–4	–	–
		C. orthopsilosis (*n* = 1)	0.125	0.03	0.5		
		C. pelliculosa (*n* = 1)	0.25	0.03	2	–	–
		C. tropicalis (*n* = 1)	0.5	8	64	1	1
	Rectal	*C. albicans* (*n* = 3)	0.25–0.5	4–16	4–32	3	2
		C. famata (*n* = 1)	1	0.25	8	–	–
		C. glabrata (*n* = 1)	0.25	0.125	2	–	–
		C. guilliermondii (*n* = 4)	0.125–0.5	0.03–16	0.5–4	1	–
		C. parapsilosis sensu stricto (*n* = 5)	0.125–0.25	0.03–0.06	0.25–2	–	–
		C. orthopsilosis (*n* = 2)	0.125	0.03	2	–	–
		C. metapsilosis (*n* = 2)	0.25	0.03	0.25–1	–	–
		C. pelliculosa (n = *2)*	0.125–0.5	0.06–0.125	2–4	–	–
		C. tropicalis (n = *1)*	0.5	16	64	1	1
Trichechus manatus	Oral	*C. albicans* (*n* = 9)	0.25–0.5	0.03–16	0.125–64	5	5
		C. famata (*n* = 2)	0.125–0.25	0.03–0.06	1–4	–	–
		C. tropicalis (*n* = 1)	0.25	0.03	32	–	1
		C. guilliermondii (*n* = 1)	0.125	0.03	2	–	–
	Nasal	*C. albicans* (*n* = 3)	0.25–1	0.03–16	0.125–64	1	1
		C. tropicalis (*n* = 1)	0.5	0.03	0.5	–	–
	Genital	*C. guilliermondii* (*n* = 1)	0.125	0.03	1	–	–
		C. tropicalis (*n* = 2)	0.25–0.5	0.03–1	0.125–2	1	–
	Rectal	*C. famata* (*n* = 2)	0.125	0.03	0.25–1		
		C. guilliermondii (*n* = 1)	0.125	0.03	1	–	–
		C. krusei (*n* = 1)	1	0.06	8	–	–

MIC minimum inhibitory concentration, *AMB* amphotericin B, *ITC* itraconazole, *FLC* fluconazole.

microorganisms (Radhouani et al. 2014; Carroll et al. 2015). Regarding bacteria, the detection of resistant strains from wild animals is associated with the interaction between these animals and human beings and their exposure to anthropic activities (Skurnik et al. 2006). Hence, in the present study, it is believed that the close interaction between manatees and human beings and the artificial environment likely contributed for the dissemination of antifungal resistant *Candida* strains.

The feeding management of the captive manatees, for instance, mostly consisting of vegetables grown for human consumption, possibly represents a potential source

Table 2. Phospholipase Production by *Candida* spp. Isolated from Sirenians.

Candida species	*T. inunguis*			*T. manatus*		
	Negative (Phz = 1)	Positive (0.64 ≤ Phz < 1)	Strongly positive (Phz < 0.64)	Negative (Phz = 1)	Positive (0.64 ≤ Phz < 1)	Strongly positive (Phz < 0.64)
C. albicans	1	33	6	–	9	3
C. ciferri	1	–	–	–	–	–
C. famata	1	–	–	3	1	–
C. glabrata	–	1	1	–	–	–
C. guilliermondii	4	4	1	2	1	–
C. krusei	–	1	–	–	1	–
C. norvegensis	1	–	–	–	–	–
C. metapsilosis	2	–	1	–	–	–
C. orthopsilosis	4	–	–	–	–	–
C. parapsilosis sensu stricto	13	1	–	–	–	–
C. pelliculosa	–	–	3	–	–	–
C. tropicalis	1	1	–	1	2	1
	28	41	12	6	14	4

Table 3. Production of Proteases by *Candida* spp. Isolated from Sirenians.

Candida species	*T. inunguis*		*T. manatus*	
	Negative (Pz = 1)	Positive (Pz < 1)	Negative (Pz = 1)	Positive (Pz < 1)
C. albicans	5	35	1	11
C. ciferri	1	–	–	–
C. famata	1	–	4	–
C. glabrata	2	–	–	–
C. guilliermondii	9	–	3	–
C. krusei	1	–	1	–
C. norvegensis	1	–	–	–
C. orthopsilosis	3	1	–	–
C. metapsilosis	3	–	–	–
C. parapsilosis sensu stricto	10	4	–	–
C. pelliculosa	3	–	–	–
C. tropicalis	1	1	3	1
Total	40	41	12	12

of resistance. The use of azole antifungals in agriculture has an important role in the induction of resistance in human and animal fungal strains, due to contact of the yeast microbiota with drug residues carried by the consumed vegetables (Müller et al. 2007; Castelo-Branco et al. 2013). Additionally, the water where the animals are kept may also represent a source of antifungal resistant strains, since it is a common vehicle for spreading chemical compounds involved in the development of resistance (Mariano et al. 2009) and it has a strong influence on the composition of the mucosal microbiota of manatees (Vergara-Parente et al. 2003). Thus, the water source for the animals and the treatment system should be carefully selected, as they directly influence the health of aquatic mammals in captivity (Arkush 2001). Finally, the high density of animals in some tanks of the surveyed institutions might also lead to higher concentrations of microorganisms in the water, facilitating the spread of

Table 4. Biofilm Production by *Candida* spp. Isolated from Sirenians.

Candida species	*T. inunguis*				*T. manatus*			
	N	W	M	S	N	W	M	S
C. albicans	32	7	–	1	3	8	–	1
C. ciferri	1	–	–	–	–	–	–	–
C. famata	1	–	–	–	4	–	–	–
C. glabrata	2	–	–	–	–	–	–	–
C. guilliermondii	6	–	2	1	3	–	–	–
C. krusei	1	–	–	–	–	–	1	–
C. norvegensis	1	–	–	–	–	–	–	–
C. metapsilosis	3	–	–	–	–	–	–	–
C. orthopsilosis	2	1	1	–	–	–	–	–
C. parapsilosis sensu stricto	7	4	3	–		–	–	–
C. pelliculosa	3	–	–	–	–	–	–	–
C. tropicalis	–	1	–	1	2	2	–	–
Total	59	13	6	3	12	10	1	1

N non-biofilm producer, *W* weak producer, *M* moderate producer, *S* strong producer.

Candida strains from one animal to another, including antifungal resistant strains.

Interestingly, a lower prevalence of resistance (20%) was observed in the isolates recovered from animals that were in captivity for less than one year, when compared to manatees that were held in captivity for longer periods: 0–5 years (42%), >5–10 years (32%), and over 10 years (39%). These findings suggest that prolonged exposure to artificial conditions may be a risk factor for the development of antifungal resistance among *Candida* spp. In addition, even though there are no published data on yeasts recovered from free-ranging sirenians, the high prevalence of *C. albicans* in the two assessed manatee species suggests that artificial conditions might influence the composition of the microbiota of captive animals, since it has been shown, through MLST analyses of human and animal strains of *C. albicans*, that this yeast species seems to be transferred from humans to wild animals (Wrobel et al. 2008). However, further studies are necessary to confirm the hypotheses on the origin and spread of antifungal resistance and on the effects of artificial conditions on the composition of the yeast microbiota of manatees, especially because the studied population is not homogenous and free-ranging manatees were not assessed in this research.

This study is also the first systematic evaluation for the detection of virulence factors in fungal strains from aquatic mammals. Considering the tested virulence attributes, production of proteases and phospholipases by the isolates of *Candida* spp. from sirenians was more prevalent than biofilm production. It is known that the production of hydrolytic enzymes and biofilms are, respectively, related to greater pathogenicity and antifungal resistance (Junqueira et al. 2012; Sarkar et al. 2014). The production of these hydrolytic enzymes, for instance, plays a central role in the fungal infective process, mainly promoting tissue damage and invasion (Ramos et al. 2015). Biofilm formation, on the other hand, is associated with refractory *Candida* infections, since these structures are associated with the development of antifungal resistance. Biofilm-forming ability of *Candida* has been associated with increased lethality among human patients with blood stream infections (Rajendran et al. 2015). Cordeiro et al. (2014) reported low production of phospholipases and high production of proteases and biofilm by *C. tropicalis* strains from veterinary sources.

The prevalence of virulence genes varies among different populations of commensal microorganisms. The human microbiota is characterized by higher prevalence of these genes, when compared to the microbiota of other animal hosts. In animals, the presence of virulence genes increases with body weight, which reveals the increased complexity of intestinal commensal microorganisms in larger species. Thus, variations in the prevalence of virulence factors among different host species may reflect a local adaptation to commensalism, rather than virulence itself (Skurnik et al. 2008; Tenaillon et al. 2010). The fact

that manatees are large herbivores that have extensive intestinal microbiota and depend on these microorganisms for the production and absorption of nutrients suggests that the detection of virulence factors in yeasts recovered from these animals may mainly be related to commensalism. The lack of reports of yeast infections in sirenians reinforces this hypothesis; however, further studies with these isolates are important to evaluate the correlation between their virulence factors and their pathogenicity to animal hosts.

Despite the considerable knowledge on the biology of some species of sirenians, there is still little information on the health aspects of these animals in many areas where they occur (Bonde and Aguirre 2004; Marsh et al. 2011). In Brazil, studies on the health of wild and captive *T. inunguis* and *T. manatus* are scarce and listed as priorities in the National Action Plan for the Conservation of Sirenia, as some subpopulations are more regionally than globally endangered (ICMBio 2011). Biomedical tests and clinical trials are of great importance for a better understanding of the population as a whole and for the implementation of management measures (Bonde et al. 2012).

The maintenance of animals in captivity for rehabilitation purposes is the basis for obtaining health information on the two species of sirenians in Brazil (D'Affonseca Neto and Vergara-Parente 2007). The recovery of *Candida* spp. from the microbiota of manatees, associated with the detection of a high rate of antifungal resistant isolates and the production of virulence factors by these yeasts broaden the spectrum of possible etiologic agents of infectious diseases in these animals and demonstrate the strong influence of artificial conditions on the health of manatees. Selective pressures within the hosts' organic systems may promote the emergence of antimicrobial resistance and virulence factors in commensal microorganisms, hence, making these hosts reservoirs of antimicrobial resistant and virulent strains (Tenaillon et al. 2010; Radhouani et al. 2014).

Considering that some of these animals, especially *T. manatus*, are kept in captivity for later release in nature, it is important to monitor the levels of antimicrobial resistance and virulence among microorganisms recovered from their natural cavities to better understand the potential long term environmental impacts and subsequent effects on wild populations. Since colonization of these animals with bacteria and fungi can be strongly influenced by artificial conditions, they can act as sources of resistance and virulence genes for the environment, conspecifics and other animal species, and rehabilitation institutions should be aware of that risk, when releasing animals back into their natural environment. Hence, the collection of biological material from free-ranging animals as well as reintroduced individuals is essential to evaluate the persistence of these microorganisms and their spreading ability in the environment.

Conclusions

Natural cavities of captive Amazonian and West Indian manatees can be colonized by resistant and virulent strains of *Candida* species, mainly *C. albicans* and *C. tropicalis*. These phenomena may have multiple origins and demonstrate the strong influence of artificial conditions on the health of animals as well as the potential environmental impacts associated with the release of manatees back into their natural habitat, as they can act as sources of resistant and virulent microorganisms for the environment, conspecifics and other animals.

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Conflict of interests

No conflict of interest declared.

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