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Bacterial flora of upper respiratory tract of captive Antillean manatees

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Abstract

The Antillean manatee (*Trichechus manatus manatus*) is classified as a 'Vulnerable' species by the International Union for the Conservation Nature and Natural Resources and is critically threatened in Brazil as a result of accidental and intentional take by humans. Infectious diseases in manatees have been reported in the scientific literature yet little is known about their inhabitants. We characterized the bacterial and fungal flora of the upper respiratory tract of eight captive Antillean manatees from two facilities in Brazil by quantifying and identifying the micro-organisms from nasal swabs. We also identified the bacteria found in the water of the facility. One group of four animals was in closed pools while the other group of two was in an open-water built into an estuary. Gram-negative bacteria, especially *Escherichia coli*, *Enterobacter sakasakii*, *Providencia rettgeri* and *Stenotrophomonas maltophilia*, predominated in manatee nasal cultures. Bacterial counts were higher in closed pools, with members of the Enterobacteriaceae prevailing (e.g., *Escherichia coli*, *Citrobacter amaloniticus*, *Enterobacter sakasakii*, and others). The prominent bacterial group founded in open-water was the nonfermentative Gram-negative bacilli (*Acinetobacter baumanni* and *Moraxella* sp.). No fungi were isolated, either in the water or the animals. The open-water facility, which possess a system of constant water renewal presented a low number of bacteria in the water samples, showed was better suited for the maintenance of the manatees in terms of water quality.

Key words: *Trichechus manatus manatus*, Antillean manatee, Brazil, captivity, bacteria and fungi, *Escherichia*, *Enterobacter*, *Providencia*, *Citrobacter*.

Introduction

The Antillean manatee (*Trichechus manatus manatus*), a coastal aquatic mammal classified as 'Vulnerable' (VU A1cd, C2a) by the International Union for Conservation of Nature and Natural Resources—IUCN (Hilton-Taylor, 2000) is critically threatened with extinction in Brazil according Brazilian Institute from Environmental and Natural Resources—IBAMA due to accidental and intentional killing by humans (Oliveira *et al*., 1990; Mignucci-Giannoni *et al*., 2000; Marine Mammal Commission, 2001; IBAMA, 2001).

Currently in Brazil, captive Antillean manatees are only found at the Units of the Aquatic Mammal Center/IBAMA, located in the States of Pernambuco and Paraíba. These animals originate from inappropriate captivities or beachings when still calves and are maintaining for national conservation purpose.

Infectious diseases caused by bacteria and fungi in manatees have been reported (Boever *et al*., 1976; Buergelt *et al*., 1984; Walsh *et al*., 1987; Forrester, 1992; Bossart, 2001), but little is known about the normal bacterial and fungal inhabitants of these animals.

Infectious diseases of the respiratory tract, especially those caused by bacteria, are the primary cause of mortality in captive aquatic mammals in

American oceanaria (Sweeney, 1986; Boness, 1997; Dunn *et al*., 2001). This is true for captive Antillean manatees in Brazil as well, where these illnesses are rarely diagnosed in time for successful treatment. This fact is directly related to the scarcity of information on the physiology of this animal species.

Thus, the objectives of this work were to characterize the bacterial and fungal inhabitants of the upper respiratory tract of Antillean manatees captive in Brazil by quantifying the colony-forming units (CFU)/plate, identifying the bacterial and fungal species, and identifying the bacteria and fungi found in the pool water.

Materials and Methods

Animals and housing

The animals chosen for the study were two males and four females Antillean manatees between 4 to 40 years old, in pools located on Itamaracá Island—Pernambuco—Brazil (7°44′52″S; 34°49′32″W). Two more animals (a male with 2 months and a female with 4 month) used in the first collection were in independent pools with separated water treatment with addition of chlorine granules. The water in these pools was completely changed twice per day and treated with addition of chlorine granules and filtered. In addition, two manatees males, with 5 and 6 years old, held in a open-water pool at Barra de Mamanguape—Paraíba—Brazil (6°50′S; 35°02′W) both facilities from the Aquatic Mammal Center of IBAMA. All the animals were clinically healthy.

Two collections of nasal swabs from the right and left nostrils were taken between May and September of 2001 at an average interval of 60 days for the animals in Paraíba and 120 days for those in Pernambuco.

In the first collection made in Pernambuco, six animals, distributed among five pools of different sizes and forms, were used. In these pools were eight manatees, two males and six females. However, only six animals were used. The other two females were exempted from the experiment due to suspected gestation, which the handling was not recommended.

The animals were handled daily and spent the night (between 16:00 to 10:00 h) at the same pool where they were fed while the water of the other pools was being treated with addition of chlorine granules, filtered, and debris aspirated (food detritus, feces, etc.). During the day, the animals had access to treated pools between 10:00 and 16:00 h.

In the second sample made in Pernambuco, the same six manatees from pools were used. However, the pair of calves that were held in the two separate pools in the first collection, were put together in a pool.

In the two collections made in Paraíba, the two manatees were held together in an open-water pool located on a branch of the Mamanguape River.

Sample collection

Holding pools were drained for sample collection and animals were physically restrained. A Hartmann nasal speculum (no. 1 in the calves or no. 3 in the adult animals) was inserted into one nostril at the time, preventing closure of the manatee's nasal orifice. Speculums were sterilized with high-temperature between collections. A sterile swab was inserted into the nostril to a depth of about 8 cm in the adult animals and about 4 cm in the calves and rotated several times.

The material from the swab was planted in 1 ml of liquid enrichment culture medium, BHI (Brain Heart Infusion), aimed at optimizing primary isolation. For the bacterial isolation, 1 µl of inoculated culture in BHI was planted on three plates containing the following culture mediums: agar containing 5% sheep blood, chocolate agar, and MacConkey agar. The plates were transferred to the Laboratory of the Medical Mycology Specialized Center, of the Federal University of Ceará, where they were incubated at 37°C for 24–48 h; the blood agar and MacConkey agar plates being incubated in a microaerophilic environment and the chocolate agar plates in an aerobic environment. Plates which not presenting growth within the incubation period (24–48 h) was considered negative. Previously was tested incubation period at 48–72 h, but we could not quantify the bacterial colonies due excessive growth with this time.

The bacterial colonies were quantified by standard plate count (colony-forming units—CFU/plate), by their morphological characteristics and grouped according Gram staining, as identified by appropriate biochemical reactions (e.g. Citrate, Voges-Proskauer, TSI, Phenylalanine deaminase, Urea hydrolysis, Indole production, Motility, Lysine decarboxylase, Arginine dihydrolase, Ornithine decarboxylase, Lactose and Sucrose fermentation, Nitrate→Nitrite, Oxidase and others).

With regard to the isolation of the fungi, one aliquot (1 µl) of inoculated culture in BHI was inoculated in three tubes containing Sabouraud agar, Sabouraud agar with chloramphenicol, and Sabouraud chloramphenicol plus cicloheximide agar. The tubes were transported to the Medical Mycology Specialized Center, where they were incubated at 25°C for 10 days. If the growth was not evidenced during the 10 days, the culture was considered negative.

Table 1. Isolated bacterial species of the upper respiratory tract of manatees of Pernambuco in Brazil.

Microorganism	Gram	No. of CFUs	
		1st Sample	2nd Sample
Acinetobacter baumanni	Negative	17	0
Acinetobacter lwoffii	Negative	130	0
Alcaligenes sp.	Negative	0	100
Cedecea lapagei	Negative	4	776
Citrobacter diversus	Negative	0	315
Citrobacter freundii	Negative	86	500
Escherichia coli	Negative	15	5523
Enterobacter agglomerans	Negative	99	520
Enterobacter sakazakii	Negative	1097	522
Enterobacter sp.	Negative	6	0
Flavobacterium sp.	Negative	31	0
Hafnia alvei	Negative	58	0
Klebsiella oxytoca	Negative	75	331
Moraxella sp.	Negative	81	0
Morganella morgani	Negative	0	6
Neisseria sicca	Negative	16	0
Oligella ureolytica	Negative	26	0
Proteus mirabilis	Negative	8	300
Providencia acalifaciens	Negative	4	0
Providencia rettgeri	Negative	10	998
Providencia stuartii	Negative	3	0
Pseudomonas aeruginosa	Negative	348	563
Pseudomonas sp.	Negative	29	0
Serratia marcescens	Negative	361	0
Serratia odorifera	Negative	76	0
Stenotrophomonas maltophilia	Negative	21	950
Bacillus sp.	Positive	391	900
Corynebacterium equi	Positive	68	0
Corynebacterium pseudodiphteriticum	Positive	59	0
Staphylococcus aureus	Positive	52	670
Staphylococcus sp.	Positive	110	0

Water samples

Water samples were collected from the captivity areas of the manatees for bacteria quantification and bacteria and fungi identification. At the Pernambuco facility, the water samples were immediately collected from the pools where the manatees spent the night. In the Paraiba facility, the water samples were collected at the same time for all animals. Collection was made with a sterile 5-ml syringe, 1 ml being transferred to a sterile test tube. From this, 1 µl was planted on plates containing a variety of culture medium. Water sample processing for bacterial and fungal isolation followed the same techniques as those described for animal specimens.

Statistical Analysis

The results were analyzed using SAS software (Statistical Analysis System, 1986 version). Differences were evaluated using the χ^2 test and Student's *t*-test.

Results

The flora present in the upper respiratory tract of captive Antillean manatees corresponded to a mixed flora consisting mainly of Gram-negative micro-organisms, 83.84% in the animals in Pernambuco (Table 1) and 66.66% for those in Paraíba (Table 2).

Regarding the differences between bacteria identified in right and left nostrils, there were no significant difference of the number of colonies and types (Student's *t*-test).

There was a significant difference between the isolated bacteria in the animals of Pernambuco

Table 2. Isolated bacterial species of the upper respiratory tract of manatees of Paraíba in Brazil.

Microorganism	Gram	No. of CFUs	
		1st Sample	2nd Sample
Acinetobacter lwoffii	Negative	130	17
Alcaligenes sp.	Negative	258	193
Budvicia aquatica	Negative	0	3
Escherichia coli	Negative	0	116
Flavobacterium sp.	Negative	10	0
Klebsiella oytoca	Negative	0	5
Moraxella sp.	Negative	62	190
Oligella ureolytica	Negative	61	0
Pseudomonas sp.	Negative	26	0
Stenotrophomonas maltophilia	Negative	0	20
Staphylococcus aureus	Positive	97	100
Staphylococcus coagulase-negativa	Positive	0	300
Staphylococcus sp.	Positive	0	8
Streptococcus sp.	Positive	0	2
Streptococcus viridans	Positive	36	0

and Paraíba ($P=0.001$). In the captive animals of Pernambuco, the bacterial micro-organisms more commonly isolated in the first sample were: *Enterobacter sakasakii*, *Bacillus* sp., *Serratia marcescens*, and *Pseudomonas aeruginosa*, totalling 66.96%. In the second sample, *Escherichia coli*, *Providencia rettgeri*, *Stenotrophomonas maltophilia*, and *Bacillus* sp., were prominent at 64.52% (Table 1). In the captive animals of Paraíba, prevalent in the first sample were: *Alcaligenes* sp., *Acinetobacter lwoffii*, *Staphylococcus aureus*, and *Moraxella* sp. at 80.44%. In the second sample, the following bacteria were prevalent: *Alcaligenes* sp., *Escherichia coli*, *Moraxella* sp., and coagulase-negative *Staphylococcus*, making up 83.86% (Table 2).

There was a significant difference between the bacteria found in the pools of Pernambuco and those in the open-water of Paraíba ($P=0.001$) in the two samples (Table 3), and the more prevalent organisms were *Escherichia coli* at 40.32%, in Pernambuco, and *Bacillus* sp. at 89.61% in Paraíba.

Following the example of the micro-organisms isolated from the animals, the bacteria found in the water of the captivity areas in Pernambuco and Paraíba were predominantly Gram-negative, with total of 75% (12 Gram-negative vs. 4 Gram-positive; Pernambuco) and 60% (3 Gram-negative vs. 2 Gram-positive; Paraíba) (Table3).

There was a no correlation ($P=0.001$) in the comparative analyses for quantification of isolated bacterial species in the animals and in the captivity water of Pernambuco, an average of 410.13 CFUs $\pm$ 278.55 ($\chi^2=1657.63$) per animal being quantified in the first sample and 1621.75 CFUs $\pm$ 829.81 ($\chi^2=4317.10$) in the second sample. In the water of the pools in the first and second samples a total of 812 CFUs and 1298 CFUs were isolated, respectively. On the other hand, when compared the bacterial species found in Pernambuco, it was shown that 75% of the species isolated in the water were also found in the animals.

The same statistical analysis was done with the data from Paraíba; however, the result cannot be considered because the sample was not significant enough for comparison due to the low number of isolated bacteria in the water, 75 CFUs in the first sample and 2 CFUs in the second sample, since in the animals the average observed was 340 CFUs (sd $\pm$ 14.14) in the first sample and 477 CFUs (sd $\pm$ 36.77) in the second sample.

In relation to the micro-organisms isolated from the animals and from the pool water, it was noted that in Pernambuco, the bacteria from the Enterobacteriaceae prevailed, while in Paraíba, the group that predominated was the non-fermentative Gram-negative bacilli (NFGB) (Table 4).

There were no fungi isolated, either in the water or the animals.

Discussion

Studies done with healthy marine mammals aimed at identifying normal inhabitants are of fundamental importance in defining handling techniques and animal treatment, because, like other mammals, the manatee can shelter a commensal microflora that does not threaten the health of the host under normal conditions (Geraci, 1991). Nevertheless,

Table 3. Bacterial species isolated from the water of the manatee captivity areas in Pernambuco and Paraíba in Brazil.

Microorganism	Gram	No. of CFUs	
		Pernambuco	Paraíba
Acinetobacter baumanni	Negative	0	1
Acinetobacter lwoffii	Negative	8	0
Alcaligines sp.	Negative	19	0
Citrobacter amaloniticus	Negative	138	0
Escherichia coli	Negative	891	0
Enterobacter cloacae	Negative	2	0
Enterobacter sakasakii	Negative	126	0
Klebsiella pneumoniae	Negative	20	0
Moraxella sp.	Negative	0	4
Proteus mirabilis	Negative	80	0
Providencia rettgeri	Negative	28	0
Pseudomonas aeruginosa	Negative	34	0
Serratia ficaria	Negative	0	1
Serratia marcescens	Negative	6	0
Serratia rubidae	Negative	4	0
Bacillus sp.	Positive	220	69
Corynebacterium pseudodiphtheriticum	Positive	347	0
Staphylococcus aureus	Positive	100	2
Staphylococcus sp.	Positive	87	0

Table 4. Bacterial group classification in captive manatees and water from facilities in Brazil.

Bacterial Group	Pernambuco		Paraíba	
	Animals	Water	Animals	Water
NFGB*	29.03%	18.75%	46.67%	40.00%
Cocci	9.68%	12.50%	33.33%	20.00%
Bacilli	9.68%	12.50%	0.00%	20.00%
Enterobacteriaceae	51.61%	56.25%	20.00%	20.00%

*Nonfermentative Gram-negative bacilli.

when the immune system or acquired resistance is harmed, as in the case of debilities, prolonged stress or medications, the animal may become susceptible to infection by certain species belonging to the normal microflora, or by other micro-organisms found in the environment (Tannock, 1995; Pelczar *et al*., 1996).

The present study was developed to characterize the bacterial and fungal contents of the upper respiratory tract of captive Antillean manatees and to investigate the bacteria and fungi present in their environment, as one of the groups of animals was in closed pools and the other was in a pool built into an estuary allowing free circulation of the water.

The bacteria isolated from the upper respiratory tract of captive Antillean manatees, as well as that identified in the pool water, for the most part, were Gram-negative, which can be attributed to the marine environment in which these animals live. According to Pelczar *et al*. (1996), the external membrane of the cell wall, which only Gram-negative bacteria possess, offers better adaptation to aquatic environments.

The differences observed between the bacterial species isolated from the animals of Paraíba and Pernambuco can be explained by environmental factors that directly influence the normal bacterial floral composition of an individual (Sharp, 1999). Manatees from Pernambuco were maintained in a closed system, where the fresh and saltwater supply from external sources was being treated with chlorine and filtration and the pools cleaned of fecal material and debris once daily. According to Howard *et al*. (1983), these procedures are insufficient for the complete elimination of bacteria and fungi.

In the research it was observed the presence of two bacteria belonging to the group of the fecal coliforms, *Escherichia coli* and the genus *Enterobacter*, both in the water samples as well as in the animals from Pernambuco. Is it important to observe the absence of this genus in the manatees and water in the Paraíba facility.

The presence of *E. coli* in the water, a bacterium of exclusively fecal origin, represents a serious health risk, depending strains are capable of provoking serious illnesses (Vieira *et al*., 1996; Soares & Maia, 1999).

Another peculiar factor was the large number of bacterial CFUs found in the collected samples from the animals and from the water of the Pernambuco facilities. This statement could be proven if compared to the pool system of Paraíba where the manatees are in an environment with free circulation of estuary water.

Besides the number of micro-organisms isolated from the Paraíba water samples being very low when compared to the pools of Pernambuco, the prevalent bacterial species in the manatees were: *Alcaligenes* sp., which is normally found in the environment (Schreckenberger & von Graevenitz, 1999), the coagulase-negative Staphylococcus group, which is one of the main components of normal human microflora (Kloos & Bannerman, 1999) and *Moraxella* sp., which is part of the normal flora of the upper respiratory tract in humans and animals, rarely causing infections (Schreckenberger & von Graevenitz, 1999). In the water samples, the *Bacillus* sp. predominated, which is a saprophytic bacterium, amply distributed in the natural environment and that is rarely associated with diseases in humans or animals (Logan & Turnbull, 1999).

Even though there are standards water quality methods for aquatic mammal, drinking water, and swimming pools, they generally do not consider the degree of bacterial pathogenicity. When used the standard counting method with plates for bacteria analysis in water, there is no particular, officially accepted number of bacteria; it depends more on their degree of pathogenicity. This method is not the same as that used to evaluate the level of contamination by fecal coliforms, which is fecal pollution of the water. It is only used to evaluate the efficiency of operations designed for the removal or destruction of organisms (i.e., sedimentation, filtration, and chlorination) (Pelczar *et al*., 1996).

Due to the expressive number of pathogenic bacteria isolated in the pools of Pernambuco, it is suspected that the water-treatment system used is insufficient to achieve an ideal water quality for the maintenance of these animals. According to Howard (1983), re-circulation, disinfecting, and filtration of water in the tanks could result in excessive bacterial growth in the water, particularly in hot climates where a fast logarithmic increase in bacteria occurs as in the waters of pools.

The pool at Paraíba, which possesses a system of constant water renewal presented a low number of bacteria in the water samples, showed was better suited for the maintenance of the manatees in terms of water quality when compared to the system used in Pernambuco.

This investigation, which gives an account of the relationship between the microbial flora in Antillean manatees and its environment, is a first-of-its-kind in Brazil, making clear the need for a complementary study which could pursue the data in a continued and deepened way, with the objective of finding alternatives which could contribute to a better quality of life for these animals.

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