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Source: Journal of Wildlife Diseases, 42(3) : 536-544

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-42.3.536

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Journal of Wildlife Diseases, 42(3), 2006, pp. 536–544
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AEROBIC MICROORGANISMS ASSOCIATED WITH FREE-RANGING BOTTLENOSE DOLPHINS IN COASTAL GULF OF MEXICO AND ATLANTIC OCEAN WATERS

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ABSTRACT: Our abilities to assess health risks to free-ranging dolphin populations, to treat live-stranded or captive dolphins, and to evaluate the risks of disease transmission between humans and dolphins have suffered from a lack of basic information on microorganisms associated with normal, presumably healthy free-ranging individuals. In order to provide these data, we sampled free-ranging bottlenose dolphins (*Tursiops truncatus*) off Florida, Texas, and North Carolina during 1990–2002. Blowhole and anal/fecal samples yielded 1,871 bacteria and yeast isolates and included 85 different species or groups of organisms. Vibrios, unidentified pseudomonads, *Escherichia coli*, *Staphylococcus* spp., and a large group of nonfermenting Gram-negative bacteria represented >50% of isolates. *Vibrio alginolyticus* and *Vibrio damsela* were the most commonly recovered bacteria from both anal/fecal and blowhole samples. Many organisms occurred sporadically in dolphins that were sampled repeatedly, but some were consistently isolated from individual animals and may indicate the carrier state. Vibrios were common, but some geographic variability in the presence of these and other organisms was noted. Potential pathogens of significance to humans and other animals were recovered.

Key words: Bottlenose dolphin, health risk, microorganisms, pathogens, *Vibrio*.

INTRODUCTION

Our abilities to assess health risks to free-ranging bottlenose dolphin (*Tursiops truncatus*) populations, to treat live-stranded or captive dolphins, and to evaluate the risks of disease transmission between humans and these animals have suffered from a lack of basic information on the microorganisms that are normally associated with this species. Obtaining these data for this and other cetacean species has proven challenging because of difficulties in obtaining samples from free-ranging animals and regulatory restraints associated with their protection. Information on microorganisms, including potentially zoonotic agents, from free-ranging cetaceans are limited to reported data from unidentified "porpoises" from the California coast (Johnston and Fung, 1969), Pacific whitesided dolphins (*Lagenorhynchus obliquidens*) and Pacific common dolphins (*Delphinus bairdi*) (Johnston and Fung, 1970), a bridled dolphin (*Stenella attenuata*) from Japanese waters (Morii, 1973), Atlantic bottlenose dolphins from the east coast of Florida (Asper and Odell, 1980, 1982) and the northern Gulf of Mexico (Carter, 1984), and unidentified species of dolphins from the Pacific near Hawaii (Palmer et al., 1989).

In addition to reported isolations from free-living species, bacteria and yeasts are reported from blowhole samples from 15 captive bottlenose dolphins sampled over a 7-yr period (Chan et al., 2001). Wong et al. (2002) found antibiotic-resistant bacteria in marine mammals. Beck and Rice (2003) reported on antibody levels in sera against several pathogenic and non-pathogenic bacteria in Atlantic ocean bottlenose dolphins. Potential human pathogens, previously unknown in marine mammals, also have been reported from

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numerous cetacean species and emphasize the need for baseline data (Varaldo et al., 1988; Neese et al., 1993; Buck and McCarthy, 1994; Foster et al., 1996; Swenshon et al., 1998; Dunn et al., 2001; Harper et al., 2002, 2003; Miller et al., 2002).

Information on normal microflora is important when attempting to rehabilitate stranded animals and may aid in the prevention of potential zoonotic disease among individuals that are involved in such efforts.

The goal of this study was to supplement existing reports related to the normal microorganisms associated with free-living cetaceans by providing a large database for microorganisms normally associated with the anus and blowhole of free-living and presumably healthy bottlenose dolphins.

MATERIALS AND METHODS

Animal capture-release

Two-hundred-forty-five free-ranging Atlantic bottlenose dolphins were captured, sampled, and released during February of 1993–94 and June of 1990–95 and 1997–2002 in, or near, Sarasota Bay, Florida (27°25′N, 82°38′W). Many were long-term residents and had been examined previously as part of an ongoing health assessment study (Wells et al., 1980; Scott et al., 1990; Wells, 1991, 2003; Wells et al., 2004). Of these 245 animals, 60 represented single samplings; 63 dolphins were sampled more than once. A total of 35 animals (all different) were captured in July 1992 in Matagorda Bay, Texas (28°30′N, 96°20′W). Twenty-one individual dolphins were sampled in July 1995 from estuarine waters near Beaufort, North Carolina (34°40′N, 76°40′W).

Microbiological sampling and culture

Samples were collected from the anus and/or blowhole, using sterile rayon swabs (Culturette II, Marion Labs, Kansas City, Missouri, USA; CultureSwab, Becton Dickinson Microbiology Systems [BDMS], Sparks, Maryland, USA; Culture C.A.T.S., Precision Dynamics Corp., San Fernando, California, USA; Transwab, Medical Wire and Equipment Co., Corsham, UK) with modified Amies or Stuart's transport medium. Fresh feces also were sampled when available; data are included with anal samples. All swabs were stored in the field under shaded, ambient conditions, and most were submitted to the laboratory within 24 hr. Samples were either processed immediately upon receiving them or were held at ambient temperature overnight before culture.

The following culture media were used: Columbia agar base with 5% defibrinated sheep blood, MacConkey agar, mannitol salt agar, thiosulfate citrate bile salts (TCBS) agar, and Sabouraud-dextrose agar with 150 mg/L chloramphenicol. The upper sections of the culture plate were inoculated directly from the swab. The remaining portions of the plate were streaked in serial fashion with a sterile loop to secure isolated colonies. Following plate inoculation, swabs were used to inoculate tubes of tryptic soy broth and Sabouraud dextrose with added antibiotic (chloramphenicol as above). Cultures were incubated under aerobic conditions at 35–37 C for up to 48 hr, and representative colonies were transferred to slants of tryptic soy agar, Sabouraud dextrose agar (bacteria and yeasts, respectively), or Marine Agar 2216 (isolates from TCBS); slants were incubated at 35–37 C for approximately 24 hr. All media were from BDMS. Early attempts to culture mycobacteria and *Brucella* spp. were unsuccessful and subsequently discontinued.

Identification of microorganisms

The Gram stain (Buck, 1982) and cytochrome oxidase (Difco Dry Slide; BDMS) reactions were recorded from slant growth. Morphology of gram-positive bacteria was determined by phase contrast microscopy. Staphylococci and streptococci/enterococci were identified to species, if possible, using the API Staph and API 20 Strep systems, respectively (bioMerieux Inc., Hazelwood, Missouri, USA). Coagulase production by staphylococci was assessed with rabbit plasma. Coryneforms were identified by morphological characteristics and catalase reaction. gram-negative isolates were identified using API 20E strips (bioMerieux). Isolates from TCBS were presumed to be *Vibrio* spp. if gram-negative, cytochrome oxidase positive, and fermentative in MOF (Lemos et al., 1985) medium. These were identified with API 20E strips and with the use of biochemical keys (Alsina and Blanch, 1994). Yeasts were identified to species, where possible, using the API 20C system (bioMerieux). In some cases, generic assignment was based on colonial morphology on corn meal agar (BDMS), carbohydrate fermentation, and assimilation of inositol and nitrate (Lodder, 1970). Confirmation of *Candida albicans* was made by observation of germ tubes in serum (Hedden and Buck, 1980).

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RESULTS

Most of the 1,871 isolates were identified to species; however, a large number (approximately 10% of total isolates) of biochemically nonreactive or weakly reactive gram-negative rods were encountered. These are designated by identifications provided in the API database and included nonspeciated isolates in the genera *Achromobacter, Acinetobacter, Alcaligenes, Moraxella, Oligella, Pasteurella*, and *Pseudomonas* (herein the AAAMOPP complex) and combinations of two or more thereof, plus species of *Chromobacterium* and *Flavobacterium.* This assemblage of nonfermenting rods may represent a portion of the normal commensal microflora of wild, healthy dolphins. Because there are marine representatives of all these genera, these isolates also may represent seawater "contaminants" from the sampling protocol. Unidentified pseudomonads also were found in large numbers (approximately 11% of all isolates) and were also difficult to identify. *Shewanella* (formerly *Pseudomonas*) *putrefaciens* was commonly recovered (3.9% of isolates) and produced H_2S and could be more easily identified.

A similar percentage of isolates (2.8%) were designated as "coryneforms," characterized as gram-positive, nonsporeforming, nonbranching, frequently pleomorphic or pallisading rods that are catalase positive. Most of these grew poorly, even on blood agar, with small, nondistinct colonies.

A number of staphylococci and streptococci/enterococci could not be identified by the API systems and were designated by genus alone, based on morphology and catalase reaction. Other than the groups noted above and a few individual vibrio isolates, serious difficulties in isolate recognition were not encountered.

The following groups of organisms (in percentage of total isolates) were recovered: vibrios (36%), enteric bacteria (17.5%), pseudomonads, including *S. putrefaciens* (13.5%), nonfermenters noted above (19%), yeasts (6.3%), staphylococci (5.8%), and streptococci/enterococci (2.2%). Together, these accounted for 91.3% of all isolates. Table 1 lists those identified microorganisms that represented at least 1% of all organisms recovered.

Eighty-five individual species or groups were recovered. Of these, two species (*Vibrio alginolyticus* [13.5%] and *Vibrio damsela* [10.3 %]) represented 23.8% of all isolates. These two species combined with unidentified *Pseudomonas* spp., *Escherichia coli*, unidentified *Staphylococcus* spp., the AAAMOPP assemblage above, *Vibrio fluvialis*, and *Vibrio parahaemolyticus* (eight of the 85 species/groups recovered) represented 54.4% of all isolates. Overall, there were 21 species or groups that individually accounted for >1% of total isolates, and combined these accounted for 78.6% of the total number of isolates.

The following species or groups individually and collectively represented <1% and 14% of total isolates, respectively: *Achromobacter/Acinetobacter/Pseudomonas* spp., *Acinetobacter/Pseudomonas* spp., *Alcaligenes/Flavobacterium/Pseudomonas* spp., *Alcaligenes* sp., *Aerococcus/Micrococcus* spp., *Cedecea* spp., *Chromobacterium* spp., *Citrobacter freundii, Citrobacter* spp., *Edwardsiella hoshinae, Eikenella/Pseudomonas* spp., *Enterobacter aerogenes, Enterobacter* spp., *Escherichia* spp., *Flavobacterium meningosepticum, Flavobacterium* spp., *Hafnia alvei, Klebsiella pneumoniae, K. rhinoscleromatis, Klyvera* spp., *Morganella morganii, Pasteurella multocida, Proteus mirabilis, P. vulgaris, Providencia alcalifaciens, P. rettgeri, P. stuartii, Pseudomonas maltophilia, P. paucimobilis, Pseudomonas/Flavobacterium* spp., *Pseudomonas* spp. (fluorescent), *Serratia liquifaciens, S. marcescens, S. odorifera, Serratia* spp., *Staphylococcus aureus* (coagulase negative), *S. epidermidis, S. hemolyticus, S. hominus, S. hyicus, Streptococcus avium, S. bovis, S. equisimilis/mitis, S. (Entero-*

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TABLE 1. Identified microorganisms (≥1% of all isolates) recovered from the anus and blowhole of bottlenose dolphins sampled in Florida, North Carolina, and Texas waters.

Isolate or group	No. of isolates	% of all isolates	% Recovery		% of animals			% of all animals
			Anus	Blowhole	Florida (n=189)	North Carolina (n=21)	Texas (n=35)	
Acinetobacter calcoaceticus var. anitratus	36.0	1.9	3.7	5.4	7	5	14	12.0
Acinetobacter calcoaceticus var. lwoffii/ Pseudomonas maltophilia	18.0	1.0	4.1	2.4	7	0	6	6.0
Acinetobacter/Pasteurella/Pseudomonas sp.	57.0	3.0	9.6	10.4	14	5	34	18.9
Aeromonas hydrophila	34.0	1.8	8.1	4.0	7	24	23	11.3
"Coryneforms"	52.0	2.8	9.2	9.1	15	14	23	17.3
Edwardsiella tarda	48.0	2.6	9.6	7.4	13	10	20	16.0
Enterobacter agglomerans	23.0	1.2	3.0	5.1	7	10	6	7.6
Enterobacter cloacae	20.0	1.1	2.2	4.7	6	5	3	6.7
Escherichia coli	130.0	7.0	41.0	6.4	43	38	11	43.2
Plesiomonas shigelloides	19.0	1.0	5.9	1.0	6	5	9	6.3
Pseudomonas paucimobilis/Pseudomonas stutzeri/ Pseudomonas sp./*Achromobacter xylosoxidans*	32.0	1.7	7.0	4.4	12	0	0	10.6
Shewanella putrefaciens	54.0	2.9	10.7	8.4	16	14	17	18.0
Vibrio alginolyticus	253.0	13.5	34.7	53.5	70	10	9	84.1
Vibrio damsela	193.0	10.3	46.9	22.2	64	29	29	64.1
Vibrio fluvialis	66.0	3.5	11.4	11.8	18	10	17	21.9
Vibrio furnissii	19.0	1.0	3.7	3.0	7	0	0	3.0
Vibrio parahaemolyticus	65.0	3.5	16.2	6.7	17	24	31	21.6
Candida albicans	21.0	1.1	4.1	3.4	5	0	0	7.0
Candida tropicalis	43.0	2.3	6.6	8.4	12	0	11	14.3
Candida sp.	22.0	1.2	3.0	4.7	7	0	0	7.3

coccus) faecalis, S. lactis, Vibrio cholerae, V. mimicus, V. vulnificus, and *Yersinia enterocolitica.* In addition, the following fungi were recovered: *Aureobasidium* sp., *Candida guilliermondii, C. lusitaniae, C. parapsilosis, C. rugosa, Rhototorua* sp., *Torulopsis (Candida) famata, T. glabrata*, and *Trichosporon cutaneum.*

Vibrio alginolyticus and *Vibrio damsela* were frequently dominant in both anal/fecal and blowhole samples. *Vibrio alginolyticus* was more frequently isolated from blowhole swabs compared with anal samples (53.5% and 34.7%, respectively), while the reverse was true for *V. damsela* (46.4% of anal samples vs. 22.2% of blowhole swabs). The identified and unidentified species of *Pseudomonas* were well represented in both types of samples as was the AAAMOPP complex and allied bacteria as well as the coryneforms, *Edwardsiella tarda, Shewanella putrefaciens*, and *Vibrio fluvialis*, with few differences noted between recoveries from anal/fecal and blowhole samples.

Several bacteria were clearly dominant in anal/fecal samples compared with blowhole recoveries and included *Aeromonas hydrophila, Escherichia coli, Plesiomonas shigelloides, Morganella morganii, Proteus* spp., *Vibrio parahaemolyticus*, and unidentified *Vibrio* species. A few isolates were more dominant in blowhole swabs: unidentifed *Pseudomonas* spp. in general, and *Streptococcus/Enterococcus* spp.

Five individual Florida dolphins were sampled on five separate occasions, and one was sampled six times. It has been noted above that some bacteria were isolated frequently from a large number of animals, for example, various vibrio and pseudomonad species, gram-negative nonfermenters, *Escherichia coli*, and *Staphylococcus* spp. Indeed, these organisms seem to occur consistently in the dolphins sampled multiple times. Most of the microorganisms recovered during the entire study appear sporadically and infrequently, associated with some animals and not with others, and not consistently in a given animal. Some seem to be found regularly only in single individuals (e.g., *Klebsiella pneumoniae* and *Candida albicans*), *Candida tropicalis* was recovered from two dolphins, and other organisms were found sporadically in a given animal (*Citrobacter freundii* in one dolphin; *Edwardsiella tarda* in three dolphins; *Vibrio fluvialis* in four dolphins).

Candida albicans was found only in Florida animals and especially in one adult female, where the yeast was isolated in all 5 yr the animal was sampled and from all anal swabs and four of five blowhole samples. It is likely that this animal exists in the carrier state. This has been demonstrated previously (Dunn et al., 1982) in a captive whale.

DISCUSSION

Vibrios were common in all three sampling areas, although *V. alginolyticus* and *V. damsela* were less frequent in Texas and North Carolina compared with Florida isolations. *Vibrio* occurrence is known to be correlated with water temperature. The enteric bacteria were frequently isolated in all samples, although *E. tarda* was more common in Texas animals while *E. coli* was isolated with lower frequency. *Morganella morgani* was recovered more often from North Carolina dolphins. These distributions may be related to the quality of the waters in which the animals were located; occurrence of enteric bacteria would be expected to be higher in inshore waters affected by land runoff. "Patchy" distributions of enteric bacteria could result from human influence in any of the areas studied. Members of the large assemblage of nonfermenting gram-negative bacteria noted above were relatively infrequently encountered in North Carolina samples, and pseudomonads were similarly rarer in Texas dolphins. Yeasts were absent in North Carolina dolphins but were common in Florida samples; only *C. tropicalis* occurred frequently in Texas

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animals and may represent the carrier state noted above.

Some gram-negative nonfermenters, staphylococci, and streptococci/enterococci may have been associated with seawater and/or human contamination during sampling. However, others are known pathogens in mammals and may be important in relation to dolphin health. These include *Citrobacter freundii, Flavobacterium meningosepticum, Hafnia alvei, Klebsiella pneumoniae, Morganella morganii, Pasteurella multocida, Proteus* spp., *Providencia* spp., *Serratia* spp., *Vibrio* spp., *Yersinia enterocolitica*, and perhaps others.

Although staphylococci have been reported in the anus of wild dolphins (Asper and Odell, 1980; Carter, 1984) and have been implicated in disease (Buck and Spotte, 1986; Palmer et al., 1988), it seemed unusual to recover them relatively frequently (4.8% of all isolates): 10.3% of anal/fecal samples and 20.9% of blowhole swabs. Although many fewer samples were from North Carolina and Texas animals than from Florida, the recovery rates were relatively similar, and, overall, 30% of all animals showed species of *Staphylococcus.* Given the increasing significance of staphylococci in human infection, their recovery from dolphins should receive additional attention in terms of more careful screening of aquarium personnel and isolates from marine mammals.

Individual animals may serve as carriers or reservoirs of certain potential pathogens such as *K. pneumoniae* or *Candida* spp. (Dunn et al., 1982). As these individual dolphins move within their own social or geographical groups and perhaps intermingle with others (e.g., in offshore areas), the possibility exists for transfer of microorganisms to other members of the community. As dolphins move from one area to another, they may introduce pathogens into new regions and perhaps new animals. For example, *Vibrio cholerae* was recovered from fecal samples from animals sampled in both Texas and Florida. As these animals move from one region to another, especially in inshore areas, large numbers of *V. cholerae* may be deposited in water and could be concentrated by bivalve shellfish.

Of particular interest was the occurrence of yeasts, especially *Candida* species and *Torulopsis (Candida) glabrata. Candida albicans, C. tropicalis, C. parapsilosis*, and *C.(T.) glabrata* are the most frequently encountered opportunistic fungi in humans (Forbes et al., 1998). In this study *C. tropicalis* occurred in 14.3% of animals, followed by unidentified *Candida* species (7.3%), *C. albicans* (7.0%), *C. famata* (3.7%), *C. lusitaniae* (3.0%), *C. guilliermondii* (1.3%), and *T. glabrata* (1.0%). In contrast to the most frequently isolated bacteria that were found in animals for all three geographical areas sampled, no yeasts were recovered from North Carolina dolphins. Perhaps this was the result of a low sample number (41 total); however, three yeasts (*C. tropicalis, C. glabrata, C. famata*) were found in relatively high frequencies (ca. 11%, 6%, and 3%, respectively) in Texas animals.

Candida albicans was found only in Florida animals and especially in one adult female where the organism was isolated in all 5 yr that the animal was sampled and from five of five anal swabs and four of five blowhole samples. It is likely that this dolphin exists in the carrier state.

Comparison with organisms isolated from 35 stranded dolphins from Gulf waters (Buck et al., 1991) shows some qualitative differences from results reported herein. Sixty-eight individual species or groups were identified in the previous study compared with 85 in this report. Quantitatively some variations were noted. In both studies (stranders and presumably healthy animals), *E. coli, Pseudomonas* spp., *V. alginolyticus*, and *V. damsela* represented >5% of the total number of isolates. In stranders, however, *E. tarda, Morganella/Proteus/Providencia* spp., and *V. parahemolyticus* were also in this group. This may indicate that, although normal animals have a wider

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spectrum of associated microorganisms in low numbers, debilitated animals are characterized by a greater number of opportunists.

Chan et al. (2001) examined 15 captive cetaceans monthly over a 7-yr period and found the following organisms as representing >2% of isolates: *Vibrio alginolyticus* (24.7%), *Candida albicans* (8.4%), *Proteus mirabilis* (6.5%), *Shewanella putrefaciens* (3.3%), *Morganella morganii* (3.1%), *Staphylococcus aureus* (2.4%), and *Pseudomonas aeruginosa* (2.1%). Our isolates (Table 1) showed respective values for the same organisms of 13.5, 1.1, 0.4, 2.9, 0.9, 0.1, and 0.8%; all except for *S. putrefaciens* were substantially lower. Fluctuations in samples over time and the occurrence of commonly isolated pathogens from blowhole samples were noted in both studies.

Asper and Odell (1980) sampled 26 wild dolphins from the east coast of Florida and found *Escherichia coli, Staphylococcus epidermidis, Proteus vulgaris*, and *Pseudomonas aeruginosa* most common in blowhole samples with frequent isolation of *Saccharomyces* species. Carter (1984) isolated a large number of different microbes from wild dolphins, but generally few organisms per animal were observed, and individual bacteria were infrequently isolated from single animals; we too noted this latter phenomenon.

Our observations indicate that there is a relatively large group of microorganisms, including potential pathogens, that exist in free-ranging coastal Gulf of Mexico and Atlantic Ocean bottlenose dolphins. The exposure of dolphins to these microbes may vary from site to site depending on a variety of environmental factors, such as seasonal changes in water temperature. In parts of the species range, water temperatures approach body temperature several months each year; in combination with local sewage effluent, this could contribute to dolphin exposure to some of the microorganisms we isolated. Many microbes are isolated frequently from blowhole or anal/fecal samples, but a suite of approximately 1–15 microorganisms are recovered with regularity. Some of these are potentially pathogenic and have the ability to cause disease or death in debilitated animals, hence the need to establish the database of commensal organisms associated with presumably healthy dolphins. When animals die and strand (or vice versa) some, perhaps many, of these "normal" microbes grow on dead tissue, and assigning a cause of death to a particular organism(s) becomes difficult, absent obvious abnormalities in internal or external structures.

Evidence has shown that certain microorganisms are frequently associated with disease and should be treated immediately. These include, but are not necessarily limited to, organisms such as *Klebsiella pneumoniae, Staphylococcus aureus* (especially coagulase positive strains), *Erysipelothrix rhusiopathiae, Pasteurella multocida, Candida albicans/glabrata*, and beta-hemolytic enterococci/streptococci (Howard et al., 1983). Other microbes such as *Pseudomonas/Shewanella* spp., *Edwardsiella tarda, Aeromonas hydrophila*, and several species of *Vibrio* are also involved in marine mammal disease. What is apparent is that there are many potential pathogens that occur in the commensal or transient state in wild populations of healthy dolphins. What circumstances lead to the transition to the disease state or stranding involvement are less clear. The establishment of a health index based on a combination of chemical and biological factors for the conservation of natural populations of protected animals should be pursued (Wells et al., 2004).

In addition, little is known of the transfer of microorganisms from animal to human, although community-acquired human infection with marine mammal–associated *Brucella* spp. has been reported (Sohn et al., 2003). Because pathogens recovered from dolphins are also dangerous to humans, it should be stressed that

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persons encountering wild populations of marine mammals should be aware of disease potential, although this is probably minimal. Equally significant, perhaps more so, is the possibility of microbial transfer from human to dolphin; virulent staphylococci are logical candidates. We concur with Beck and Rice (2003) that continued monitoring of the health of individual dolphins will provide relevant supplemental medical information.

ACKNOWLEDGMENTS

This work was supported in part by the National Marine Fisheries Service, Mote Marine Laboratory, Earthwatch Institute, Dolphin Quest, The Disney Wildlife Conservation Fund, and Dolphin Biology Research Institute. Special gratitude is extended to Michael Scott, Blair Irvine, Jay Sweeney, Forrest Townsend, Dave Casper, Rae Stone, Larry Fulford, Graham Worthy, Sue Hofmann, Kim Urian, Stephanie Nowacek, Brian Balmer, and the many volunteers working with the Sarasota Dolphin Research Program and the National Marine Fisheries Service for their help in collecting samples. Charles Manire provided samples from Mote Marine Laboratory's Dolphin and Whale Hospital. Sample collection in Florida was accomplished under Scientific Research Permit 655, 945, and 522-1569 issued by the National Marine Fisheries Service. We are very grateful for reviews of drafts of the manuscript by Debbie Fauquier.

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Received for publication 23 November 2004.

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