Aquatic Mammals 2007, *33*(2), 185-194, DOI 10.1578/AM.33.2.2007.185

Prevalence and Diversity of Antibiotic Resistant *Escherichia coli* in Bottlenose Dolphins (*Tursiops truncatus*) from the Indian River Lagoon, Florida, and Charleston Harbor Area, South Carolina

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Abstract

A total of 724 *Escherichia coli* isolates sampled from 38 wild bottlenose dolphins (*Tursiops truncatus*) from the Charleston Harbor area, South Carolina, and the Indian River Lagoon, Florida, were screened for resistance to 25 antibiotics. The percentages of animals harboring at least one resistant isolate differed significantly between sampling locations. No resistance was detected in *E. coli* from dolphins at either site for six of the 25 antibiotics tested. Resistance to penicillin was most common followed by cephalothin, ampicillin, and amoxicillin. Within-animal isolate variability was examined in addition to between sampling locale. Isolates from animals sampled in the Charleston Harbor area exhibited a greater complexity of resistance patterns and within individual diversity compared to isolates sampled from animals in the Indian River Lagoon. Causes related to the observed heterogeneity are discussed.

Key Words: bottlenose dolphin, *Tursiops truncatus*, antibiotic resistance, *E. coli*, Charleston Harbor Area, Indian River Lagoon

Introduction

A great deal of attention has been directed at the emergence of bacterial strains resistant to antimicrobial agents. It is thought that the widespread use of antibiotics has allowed for the development of resistance strains by exerting positive selective pressure on bacteria-carrying genotypes conferring resistance (Koshland, 1994). Once established, bacteria can pass genetic material conferring resistance through a variety of well-studied mechanisms (e.g., conjugation, transformation, and transduction), allowing for the proliferation of these traits through the bacterial population in addition to the potential for cross-species transmission. Current consensus opinion links the widespread use of antimicrobials for therapeutic use to the recent increase of bacterial strains resistant to antimicrobials, while the effect of the use of antimicrobials as growth promoters in commercial livestock, poultry, and fisheries operations in relation to the emergence of resistant bacterial strains is currently under debate (Nue, 1992; Prescott et al., 2000; McEwen & Fedorka-Cray, 2002; Phillips et al., 2004).

Recently, attention has been focused on the fate of antimicrobials in the environment in addition to the direct release of bacterial strains resistant to antimicrobial agents. Unused antimicrobial therapeutics are often disposed into sewage systems or are unmetabolized during treatment and are discharged into wastewater (Kummerer, 2001, 2004). For example, numerous studies have detected antibiotics in low-level concentrations (ranging from µg to ng per liter) in effluent from sewage treatment plants and hospitals (Golet et al., 2001; Kummerer, 2003). There is additional evidence suggesting that many antimicrobial agents remain active in the environment due to an inability to be biodegraded (Al-Ahmad et al., 1999). Antibiotic resistant bacteria have been isolated from a variety of aquatic sources, ranging from sewage effluent to drinking/ground water (Baya et al., 1986; Kasper et al., 1990; McKeon et al., 1995). The presence of bacteria resistant to antimicrobial agents is so ubiquitous that multidrug resistance (MDR) patterns have been used in the development of source tracking assays of fecal pollution detection (Wiggins, 1996; Hagedorn et al., 1999; Harwood et al., 2000). As our understanding of mechanisms by which antimicrobial agents and resistant strains enter into the environment increases, so does our need to examine their

effects on and transport through different trophic levels of the ecosystem.

While the occurrence of bacteria resistant to antimicrobials in wild animals has been well-documented, the prevalence and ubiquity of these events remain largely inconsistent. For example, high levels of antibiotic resistance were observed in bacteria isolated from wild rodents from northwest England (Gilliver et al., 1999), while low rates of bacteria resistant to antimicrobials have been reported in moose, deer, and voles from Finland, an area where historical antibiotic usage and mean number of inhabitants is less than the United Kingdom (Osterblad et al., 2001). These findings lead to the postulation of an association between an increase in the prevalence of antibiotic resistant bacteria present in wildlife with proximity to human or anthropogenic effects (Gilliver et al., 2001). Recent work on birds from South America, however, has questioned the general premise that the genesis of antibiotic resistant bacteria is predominantly via anthropogenic effects as a large number of bacterial isolates carrying resistance to antibiotics were observed in birds living in remote areas (Nascimento et al., 2003). The majority of work on wildlife has focused on terrestrial animal populations and has been largely limited for within-individual sampling, focusing on surveying a small number of isolates of the different enterogenic microbes typically found in the targeted species versus a more rigorous examination of diversity within a particular species of bacterium. Typically, data are analyzed using discriminate analysis with the intent to categorize the profile of resistance to assign source identity. Questions regarding the proportion of bacteria carrying resistance within a host and the diversity among these isolates remain largely unaddressed. With regard to marine systems, marine mammals have been proposed as candidates to serve as monitors for ecosystem health (Marine Mammal Commission, 1999; Reddy et al., 2001; Wells et al., 2004). Bottlenose dolphins (*Tursiops truncatus*) have been identified as a suitable species to serve as a model for examining environmental contaminant effects in marine mammals (Holden, 1972; Aguilar & Borrell, 1994) in addition to being marine ecosystem health indicators (Marine Mammal Commission, 1999; Bossart, 2006). They are long-lived, apex predators resident to coastal waters in temperate and tropical areas, making them well-suited as sentinels for detection of environmental stressors (Wells et al., 2004).

The objective of the current study was to investigate the prevalence and diversity of antibiotic resistant gastrointestinal *Escherichia coli* (*E. coli*), a bacterium common to mammals and routinely used for identification of fecal coliform pollution in aquatic systems, isolated from wild bottlenose dolphins from two distinct locations along the southeastern Atlantic coast of the United States. We examine whether the prevalence of antibiotic resistant *E. coli* between sampling sites was homogenous and apply a population genetic analysis to the collected data in an effort to estimate within-animal (individual) isolate diversity and elucidate differences in resistance profiles among animals from discrete sampling sites.

Materials and Methods

Study Areas

Capture of dolphins was conducted in the summer of 2003 during a comprehensive health assessment conducted by the Harbor Branch Oceanographic Institution, Fort Pierce, Florida, and National Ocean Service (NOS) Center for Coastal Environmental Health and Bimolecular Research (CCEHBR), Charleston, South Carolina (National Marine Fisheries Permit No. 998-1678-00). Animals were sampled from two distinct sites: Indian River Lagoon, Florida (IRL) (Figures 1A & 1B), and Charleston Harbor area, South Carolina (CHS) (Figure 2). The IRL is an aggregate of three estuarine bodies of water and extends 260 km along Florida's eastern central coast from Ponce De Leon inlet in the north to Jupiter Inlet in the south. The region is characterized by a mix of residential, urban, agricultural, and undeveloped areas. Flushing of the system is low due to restricted connectivity to the Atlantic Ocean, limited by five inlets and one lock. The Charleston Harbor estuary extends over approximately 3,300 km^2 (Yassuda et al., 2000). Two of the area's primary tributaries—the Ashley and Wando—are tidal sloughs with limited freshwater input, whereas the third—the Cooper River—has an average freshwater flow of 140 to 170 $m^3 s^{-1}$. The region is characterized by mixed residential, urban, and light industrial use.

Sample Collection

Dolphin fecal samples were collected via rectal swab in Aimes transport media (MML Diagnostics Packaging, Inc., Troutdale, OR) or a direct fecal sample placed in a sterile tube. Samples collected in the IRL were stored in coolers with ice packs and shipped cold overnight to the CCEHBR laboratory. Charleston Harbor samples were placed in coolers with ice packs and delivered same day to the CCEHBR laboratory. All samples were immediately stored at 4° C upon arrival.

Isolation of E. coli

Swabs and fecal samples were streaked to Difco TM Violet Red Bile (VBR) Agar (Becton,

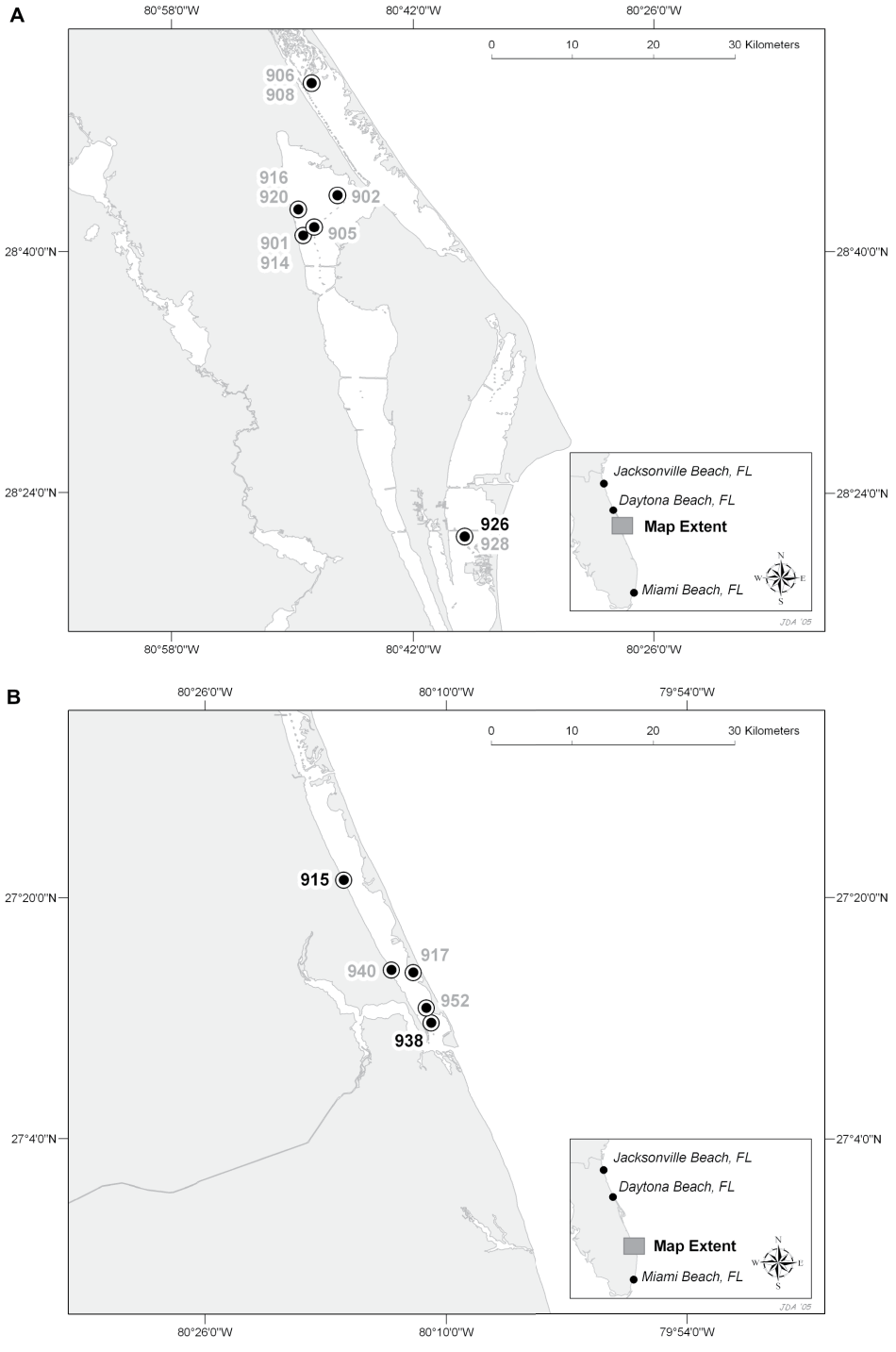

Figure 1. Maps of the northern (A) and southern (B) regions of the Indian River Lagoon (IRL), Florida, study site showing identification number of individuals sampled; identification numbers in bold represent individuals sampled that were harboring antibiotic resistance bacteria.

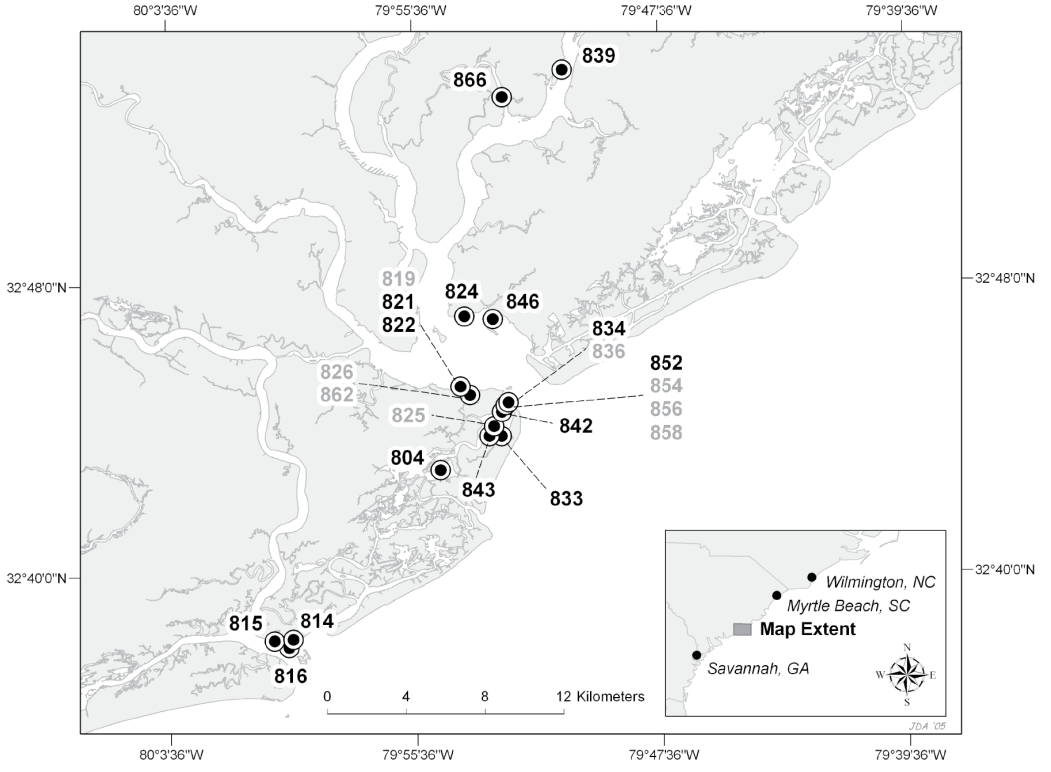

Figure 2. Map of the Charleston Harbor and surrounding waters from the South Carolina study site showing identification number of individuals sampled; identification numbers in bold represent individuals sampled that were harboring antibiotic resistance bacteria.

Dickerson and Company, Sparks, MD) within 24 h of collection and incubated overnight at 37° C. Isolated purple/red colonies indicative of *E. coli* were picked and patched to Difco™ Nutrient Agar with 4-methylumbelliferyl-B-D-glucuronide (MUG) (Becton, Dickerson and Company, Sparks, MD) and incubated at 37° C for 18 to 24 h. A secondary screening was conducted using UV excitation to ensure colonies were *E. coli.* Isolated fluorescing colonies were marked, transferred to a second MUG plate, incubated overnight at 37° C, and used the next day for antibiotic screening. Colonies not assayed next day were picked and placed in Tryptic Soy Broth containing 15 to 20% glycerol, vortexed, frozen at -80° C and assayed at a later date. A target sample number of 20 individual isolates per animal was selected but not always achieved. Samples where less than 10 isolates were obtained were excluded from analysis.

Antibiotic Resistance Screening

Colonies were assayed directly for resistance to 25 antibiotics using a customized Dade Behring Microscan Panel (Dade Behring Inc., Deerfield, IL). Panels were in a 96-well format with varying concentrations of dehydrated antibiotics and two antibiotic-free control wells. Antibiotics and the concentrations used are presented in Table 1. Individual colonies were picked and used as inoculum following a turbidity standard technique by Dade Behring. Panel rehydration and inoculation were performed using the RENOK® system and disposable inoculators. After inoculation, panels were incubated overnight at 37° C and read the following morning. Panels were read manually and results recorded following Dade Behring instructions with the following exception: growth in treatment wells was determined positive if the sample achieved approximately 80% comparative growth to antibiotic-free control wells. Isolates were typed as resistant by growth in either of the highest two concentrations of the antibiotic tested. Individuals were scored as harboring antibiotic resistance bacteria if any isolate was scored as resistant to one or more of the 25 antibiotics tested.

Table 1. Antibiotics, concentrations, and detection of resistance strains of *E. coli* collected from dolphins in the Indian River Lagoon and Charleston Harbor study sites; antibiotic codes are given in parentheses. Concentrations where growth was detected and classified as resistant are in bold.

		Detected	
Antibiotic	Concentrations tested (µg)	IRL	CHS
Penicillins			
Penicillin (P)	8, 16, 32, **64**, > 64	+	+
Ampicillin (Am)	4, 8, 16, **32**, > 32	+	+
Amoxicillin (Amx)	4, 8, 16, **32**, > 32	-	+
Cephems			
Cephalothin (Cf)	4, 8, 16, **32**, > 32	+	+
Ceftriaxone (Cax)	8, 16, **32**, > 32	-	+
Cefoxitin (Cfx)	8, 16, **32**, > 32	-	+
Carbapenems			
Meropenem (Mer)	2, 4, 8, **16**, > 16	-	-
Imipenem (Imp)	2, 4, 8, **16**, > 16	-	+
Folate Pathway Inhibitors			
Sulfathiazole (Sz)	250, **500**, > 500	-	+
Trimethoprim (T)	2, 4, **8**, > 8	-	+
Trimethoprim/Sulfamethoxazole (T/S)	2/38, **4/76**, > 4/76	-	+
Quinolones and Fluoroquinolones			
Moxifloxacin (Mox)	1, 2, 4, **8**, > 8	-	+
Nalidixic Acid (NA)	4, 8, 16, **32**, > 32	-	-
Ciprofloxacin (Cp)	1, 2, **4**, > 4	-	-
Ofloxacin (Ofl)	1, 2, 4, **8**, > 8	-	+
Aminoglycosides			
Amikacin (Ak)	8, 16, 32, **64**, > 64	-	-
Gentamicin (Gm)	2, 4, 8, **16**, > 16	-	-
Streptomycin (St)	16, 32, 64, **128**, > 128	-	+
Apramycin (Apr)	8, 16, **32**, > 32	-	+
Tetracyclines			
Tetracycline (Te)	4, 8, 16, **32**, > 32	-	+
Oxytetracycline (Otet)	4, 8, 16, **32**, > 32	-	+
Macrolides			
Erythromycin (E)	4, 8, 16, **32**, > 32	-	+
Azithromycin (Azi)	1, 2, **4**, > 4	-	+
Phenicols			
Chloramphenicol (C)	8, 16, **32**, > 32	-	+
Nitrofurantoins			
Nitrofurantoin (Fd)	4, 8, 16, **32**, > 32	+	+

Statistical Analysis

Differences in the proportion of individuals carrying antibiotic resistance bacteria between sampling locations were compared using a goodness of fit test (G-test). To address patterns of within-animal isolate diversity and to partition resistance variance, we conducted an analysis of molecular variance (AMOVA) by coding isolate resistance pattern data into phenotypic haplotypes from each locale (Nei, 1987; Excoffier et al., 1992). Isolates showing no resistance were considered a single haplotype as was each different antibiotic resistance profile. The resulting transformations led to defining a total of 36 bacterial haplotypes. Standard genetic diversity estimates were calculated from transformed haplotypes as implemented in the computer program *Arlequin*, Version 2.0 (Genetics and Biometery Laboratory, University of Geneva, Switzerland) to examine within-animal bacterial phenotypic

diversity (Nei, 1987). Similarly, conventional F-statistics from haplotype frequency data were used in the AMOVA analyses as implemented in *Arlequin* where isolates from single individuals comprised the "population" data class.

Results

Dolphin fecal samples or rectal swabs were collected from 39 animals from CHS. *E. coli* was successfully isolated from 27 animals (69%), of which 23, comprising 432 isolates, were successfully screened for antibiotic resistance. Of these, 108 isolates (25%) from 15 animals (65%) harbored at least one *E. coli* isolate resistant to one or more of the following antibiotics: amoxicillin, azithromycin, cefoxitin, cephalothin, chloramphenicol, ciprofloxacin, erythromycin, imipenem, nitrofurantoin, ofloxacin, oxytetracycline, penicillin, streptomycin, sulfathiazole, tetracycline, trimethoprim, and trimethoprim/sulfamethoxazole (see Table 2). The average number of isolates assayed per animal from CHS was 19, with a range from 13 to 20.

For the IRL, fecal samples or rectal swabs were collected and processed for *E. coli* isolation from 33 animals. *E. coli* was successfully isolated from 16 animals (48%), of which 15, comprising 284 isolates, were successfully screened for antibiotic resistance. Resistance to one or more antibiotics was observed in 25 isolates (8.8%) collected from three animals (20%) and included penicillin, amoxicillin, cephalothin, and nitrofurantoin. Of the 15 animals sampled in IRL, the average number of *E. coli* isolates examined per animal were 19, with a range from 16 to 20. No resistance was detected from either site for six of the 26 antibiotics tested: meropenem, moxifloxacin, nalidixic acid, ciprofloxacin, amikacin, and gentamicin. For pooled data from both sites, resistance to penicillin (34%) was most common followed by cephalothin (29%), ampicillin (26%), and amoxicillin (24%).

Goodness of fit tests conducted on percentages of animals harboring at least one resistant isolate revealed significant differences between sampling locations (Gadj = 7.43, $p < 0.01$). From the AMOVA analyses, it was found that the samples from CHS animals had a higher percentage of the variation (60%), which may be explained by differences within individuals (among resistant haplotypes), whereas for the IRL, differences among individuals (those harboring antibiotic resistant *E. coli* vs those that do not) accounted for the greater percentage of variation (63%). Similarly, the range of estimates of phenotypic diversity was the greatest in animals sampled in CHS, where the lowest and highest values were recorded.

Discussion

For the 38 animals successfully screened for antibiotic resistant *E. coli* in this study, 18 (47%) had bacterial isolates resistant to one or more antibiotics. Prevalence of animals harboring resistant bacteria was significantly higher in CHS vs the IRL. Similarly, within-animal diversity of bacterial phenotypes was greatest in CHS animals where resistance to 20 antibiotics was documented. In contrast, resistance to only five antibiotics was observed from animals sampled in the IRL. Resistance to the same five antibiotics was observed in samples obtained from CHS animals and four of these five antibiotics were the most commonly observed: penicillin, cephalothin, ampicillin, and amoxicillin.

Resistance patterns differed noticeably between the CHS and IRL sampling sites, with more complex, resistant phenotypes being observed from isolates sampled from CHS animals. As observed in the AMOVA results, these data also reflect greater within-animal bacterial isolate variability (in regard to resistance profiles) from CHS than the IRL. The greater among-individual variability observed in animals sampled in the IRL is likely due to the homogenization effect of the large proportion of sampled isolates without antimicrobial resistance. As such, the heterogeneity in resistance profiles observed between sampling sites may reflect differences in regional selective pressure or exposures.

It is difficult to ascertain the exact mechanism by which the observed resistance to antimicrobial agents has been acquired, and it is likely caused by multiple factors. Generally, the spread of genes conferring antibiotic resistance is thought to be derived from either horizontal gene transfer or genetic change via mutation (Silva, 1996; Baquero & Blazquez, 1997). Potential introduction of both antimicrobial agents and resistant microbes to the watershed and, ultimately, the marine environment includes sewage (e.g., septic tank seepage), sewage treatment plants, and runoff (Kummerer, 2003). Microbial antibiotic resistance data for *E. coli* from wastewater treatment plants in close proximity to Charleston (Hilton Head Island) identified resistance to penicillin and ampicillin as the two most common (Webster et al., 2004). It was further noted that there was an increase in the prevalence and complexity of antibiotic resistant bacteria isolated in developed areas of the watershed compared to rural areas (Webster et al., 2004). These data suggest a potential source for resistance bacteria and their subsequent establishment into dolphin intestinal fauna. There are four wastewater treatment plants that discharge into the Charleston Harbor, with an additional

Table 2. Number of *E. coli* isolates screened for resistance, number, and type of antibiotic resistance haplotypes observed; and phenotypic diversity estimates in dolphins sampled from Charleston Harbor (ID 800 series) and the Indian River Lagoon (ID 900 series).

Dolphin ID	Number isolates	Number resistant	Resistance patterns	Phenotypic diversity
804	20	1 (5%)	1-P	0.10 ± 0.08
814	20	16 (80%)	1-C, Cf, E; 5-Cf, E; 10-E	0.68 ± 0.07
815	20	3 (15%)	2-E; 1-St	0.27 ± 0.12
816	20	10 (50%)	1-E, Imp; 9-E	0.57 ± 0.06
821	16	2 (13%)	2-Am, Amx, P, Sz, T, T/S	0.23 ± 0.13
822	15	8 (53%)	1-Am, Amx, C, Cf, E, P; 1-E, Otet, Te; 1-E, Otet, Sz, Te; 1-Cf; 2-E; 2-Otet, Te	0.78 ± 0.10
824	19	5 (26%)	1-Am, Amx, Otet, P, SzT, T/S; 1-Am, Amx, Cf, Otet, P, St, Sz, T, T/S; 1-Otet, Sz, Te; 2-Am, Amx, P, Sz	0.46 ± 0.14
833	20	7 (40%)	1-Am, Amx, C, Cf, Cfx, P, T, T/S; 1-Am, Amx, Cf, Cfx, Fd, P, T; 1-Am, Azi, Cf, Cfx, Fd, P, T, E; 1-Cfx, P; 2-E; 2-P	0.64 ± 0.12
834	20	1 (5%)	1-Am	0.10 ± 0.09
839	20	16 (80%)	14-Am, Amx, Cf, Cfx, P; 1-Am, Amx, Cf, Cfx, P, Ofl; 1-Am, Amx, Cax, Cf, Cfx, P	0.49 ± 0.12
842	20	11 (55%)	1-Am, Apr, E, Otet, T, T/S, St; 1-Am, Amx, Apr, Azi, Cf, Otet, P, St, Sz, T, T/S; 1-Am, Amx, Apr, Azi, Otet, P, Sz, T, T/S; 2-Am, Amx, Apr, Otet, P, C, Sz, T, T/S; 2-Am, Amx, Apr, Otet, P, T, T/S; 4-Am, Amx, Apr, Otet, P, Sz, T, T/S	0.77 ± 0.08
843	20	3 (15%)	1-T; 2-Am, Amx, Otet, P, Sz, T, T/S	0.28 ± 0.12
846	20	6 (30%)	1-Sz, T, T/S; 2-Cf, P; 3-Cf	0.50 ± 0.12
852	20	1 (5%)	1-Cf, P	0.10 ± 0.09
866	20	19 (95%)	19-Am, Amx, Cf, Cfx, P	0.10 ± 0.09
915	16	1 (6%)	1P	0.13 ± 0.11
926	20	19 (95%)	3-Am, Amx, Cf, P; 15-Am, Amx, P; 1- Am, P	0.43 ± 0.13
938	16	5 (31%)	5-Fd, Cf	0.46 ± 0.10

six that discharge into the surrounding Ashley, Wando, and Cooper Rivers or their tributaries (SCDHEC, 1998). Similarly, there are five major hospitals operating within or near the city of Charleston. Thus, there are numerous potential sources for both low-level chronic exposures to antimicrobial agents and bacteria strains resistant to antimicrobials.

The IRL site represents a much more diverse environment. The narrow and long lagoon runs roughly 260 km through two coastal counties with varying developmental pressures. The northern portion of the IRL (Mosquito Lagoon and the northern extreme of the Indian River) is buffered by the Merritt Island National Wildlife Refuge where development has largely been curtailed, while the remaining watersheds that feed into the IRL comprise one of the fastest-growing population centers in the United States (Phlips et al., 2002). Since 1996, wastewater treatment plant discharge into the lagoon has been greatly limited as most treatment plants currently utilize either percolation ponds or subsurface drain fields (Sigua et al., 2000; Barile, 2004). Issues regarding urban, agricultural, and wastewater treatment runoff continue to exist, however, as investigations on nutrient loading into the IRL from these systems appears to be substantial (Barile, 2004). Generally, water quality in the northern IRL is considered good, but it decreases in proximity to increasing development (e.g., urban areas of Titusville and Cocoa) (Sigua et al., 2000). There are 19 wastewater treatment plants located along the barrier island separating the IRL from the Atlantic Ocean, with numerous additional facilities located on the Florida mainland supporting the larger urban and residential areas (e.g., Melbourne, Cocoa, and Titusville) (Barile, 2004). Thus, similar to the Charleston site, numerous potential sources for introduction of antimicrobial resistant microbes exist in the IRL. It is interesting to note, however, that of the ten animals sampled in the northern IRL, eight with no observed bacteria-harboring resistance to antibiotics were sampled in the extreme northern portion of the IRL (bordering the Merritt Island National Wildlife Refuge). The remaining two (one of which contained antibiotic resistant bacteria) were sampled near the urbanized Cocoa Beach area. Similarly, two of the five southern IRL animals harboring antibiotic resistant bacteria were sampled in close proximity to Stuart and the St. Lucie River, also an urbanized area. These data suggest that the home ranges of dolphins from these areas may be limited and are in concordance with previously published reports on the movement of dolphins from similar ecosystems (Odell & Asper, 1990; Gubbins, 2002). As the behavior of the animal contributes significantly to the potential hazards it is ultimately exposed to, we are planning to incorporate photo-identification studies in an effort to define core habitat use.

It appears that the within-region variability observed in the IRL may be an important component in our understanding of the dynamics of the system due to the apparent heterogeneity of animals sampled harboring resistant isolates (see Figure 1). We recognize that it is likely that the current pooling of samples from the southern and northern extremes of the IRL may constitute a mixing of local populations. Unfortunately, small sample size within subregions of the IRL precludes a robust within-IRL analysis. In light of these limitations, we are continuing to sample these areas to increase our sample size and better examine within-IRL variability. Regardless, it is compelling to consider a positive association between urban development (decreased water quality) and the presence of dolphins harboring antibiotic resistance bacteria. These associations are beyond the scope of this study, however, and will have to wait further investigations.

We are continuing to screen intestinal *E. coli* isolate samples for antibiotic resistance in an effort to examine temporal and within-region effects (particularly within the IRL). We are also planning molecular assays to examine isolate genetic diversity to increase confidence in the identification of potential sources for resistant strain introduction. The effects of the high prevalence of intestinal bacteria resistant to antimicrobials in inshore dolphin populations (particularly in CHS) on the ecosystem at this point in time is largely speculative. Our data suggest, however, that these animals may be serving as reservoirs for antibiotic resistant bacteria and quite possibly acting as vectors for dissemination into other environments.

Acknowledgments

The Bottlenose Dolphin Health and Risk Assessment Project is a collaborative effort between the National Oceanic and Atmospheric Administration (NOAA), the National Ocean Service Center for Coastal Environmental and Biomolecular Research, and the Harbor Branch Oceanographic Institution (HBOI). We would like to thank the numerous researchers who participated in the capture and release field study of dolphins in South Carolina and Florida. We are especially thankful to Forrest Townsend, Larry Fulford, Larry Hansen, Eric Zolman, Steve McCulloch, the NOAA and HBOI staff, all of the veterinarians who provided their expertise, and the volunteers whose help made the health assessment studies possible. The authors thank Jeffery Adams, Thomas Pullaro, and Laura Webster for reviewing earlier drafts of this manuscript. A special thanks to Jeffery Adams for assisting with

figures and Lori Schwacke for comments and discussions. This study was conducted under National Marine Fisheries Permit No. 998-1678-00, which was issued to Gregory Bossart of Harbor Branch Oceanographic Institution in March 2003. This study was supported through the NOAA Fisheries Marine Mammal Health and Stranding Response Program, NOAA National Centers for Coastal Ocean Science, and also the Florida Protect Wild Dolphins License Plate Fund.

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