RESEARCH ARTICLE

Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast

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Abstract

The prevalence of antibiotic-resistant bacteria in the marine environment is a growing concern, but the degree to which marine mammals, seabirds and fish harbor these organisms is not well documented. This project sought to identify the occurrence and patterns of antibiotic resistance in bacteria isolated from vertebrates of coastal waters in the northeastern United States. Four hundred and seventy-two isolates of clinical interest were tested for resistance to a suite of 16 antibiotics. Fifty-eight percent were resistant to at least one antibiotic, while 43% were resistant to multiple antibiotics. A multiple antibiotic resistance index value ≥ 0.2 was observed in 38% of the resistant pathogens, suggesting exposure of the animals to bacteria from significantly contaminated sites. Groups of antibiotics were identified for which bacterial resistance commonly co-occurred. Antibiotic resistance was more widespread in bacteria isolated from seabirds than marine mammals, and was more widespread in stranded or bycaught marine mammals than live marine mammals. Structuring of resistance patterns based on sample type (live/stranded/bycaught) but not animal group (mammal/bird/fish) was observed. These data indicate that antibiotic resistance is widespread in marine vertebrates, and they may be important reservoirs of antibiotic-resistant bacteria in the marine environment.

Introduction

The promise of bacterial disease control through the discovery and use of antibiotics has been dramatically undermined by the appearance of resistant strains and the spread of resistance genes. A background level of antibiotic resistance occurs naturally in any environment, and as such, antibiotic resistance genes and antibiotic resistant microorganisms have been documented in areas with little to no obvious anthropogenic impact or influence, and in environmental samples obtained before the use of antibiotics in disease treatment (Graves *et al.*, 1988; Magee & Quinn, 1991; McKeon *et al.*, 1995; Boon & Cattanach, 1999; Pei *et al.*, 2006; Singer *et al.*, 2006; Sjolund *et al.*, 2008). However, the widespread use of these drugs in human disease treatment and agriculture has resulted in a significant increase in the spread and persistence of antibiotic resistance in the environment (Smith *et al.*, 2002).

Steps have been taken to reduce the dissemination of antibiotics into the environment, such as limiting the amount and types of antibiotics used (Shlaes *et al.*, 1997). However, recent work has shown that even after the removal of the selective pressure of antibiotics in an environment, resistance levels have been slow to decline (Heuer *et al.*, 2002; Sørum *et al.*, 2006) or have even increased (Enne *et al.*, 2001). The dogma that maintenance of resistance genes in the absence of selection is energetically costly for an organism has become less accepted as studies have shown that some organisms carrying resistance genes in a population are actually quite robust (Salyers & Amabile-Cuevas, 1997; Singer *et al.*, 2006). Additionally, metal pollution has been demonstrated to indirectly aid in the long-term persistence

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of antibiotic resistance in bacterial communities due to a combination of the stability of metals in terrestrial and aquatic environments and commonly occurring co- and cross-resistance to metal toxicity and antibiotics (Rasmussen & Sorenson, 1998; Baker-Austin *et al.*, 2006; Wright *et al.*, 2006; De & Ramaiah, 2007).

The persistence and spread of bacteria resistant to antibiotics in the environment is of concern because of the potential increase in community-acquired resistances. This is in contrast to the traditional antibiotic-resistance hot spots of hospitals and nursing homes where close physical proximity and people highly susceptible to infection are thought to contribute to the spread of resistant bacteria. Antimicrobial use in treatment of humans and food-animal husbandry (terrestrial and aquatic) results in the release of wastes that carry both antibiotics and antibiotic-resistant (ABR) bacteria into the terrestrial and coastal marine environment (Silbergeld *et al.*, 2008). Recent analysis of soil and aquatic metagenomic databases suggest that antibiotic resistance is diverse and widespread in environmental bacteria (D'Costa *et al.*, 2007). There are only a few reports of antibiotic resistance in marine animals compared with terrestrial animals, but it has significance with regard to marine mammal stranding and rehabilitation activities, and dissemination of resistant bacteria in the environment. To date, studies that examined the prevalence and types of antibiotic resistance in bacteria isolated from marine organisms have included surveys of seabirds in rehabilitation facilities (Ziegerer *et al.*, 2002; Steele *et al.*, 2005), stranded seals (Johnson *et al.*, 1998) and sharks (Blackburn, 2003). All of these studies have shown that ABR bacteria were consistently recovered from a variety of animals, and that usually more than half of the isolates (sometimes as many as 75%) were resistant to at least one antibiotic.

While those studies were successful in identifying and describing the presence of ABR bacteria in marine animals, the number of isolates and samples tested were not usually large enough to allow statistical evaluation of the data. With the potential for marine animals to serve as reservoirs for both pathogenic and commensal ABR bacteria, a more comprehensive study is needed to thoroughly document the occurrence of ABR bacteria in marine mammals and seabirds, and determine whether there are patterns to the presence and persistence of resistant bacteria. We investigated differences in ABR bacteria type and occurrence among different mammals and seabirds, and whether different animal provenance affected the types of ABR patterns present. This project was designed to examine patterns associated with ABR in marine animals by surveying bacterial isolates recovered from stranded, bycaught and live marine mammals (seals, whales, dolphins and porpoises) and seabirds in the Northeastern United States.

Materials and methods

Bacterial culture and antibiotic sensitivity testing

As part of a broader survey of pathogens in coastal marine vertebrates (Bogomolni *et al.*, 2008), tissues/organs routinely sampled included fecal/cloaca swabs for live animals, and thorax and abdomen or coelom for those examined by necropsy. Swabs from nasal/blowhole/nares were collected as appropriate and practical on live animals and if contamination of the outside surface of dead animals was minimal. Other sites were chosen for bacterial isolation if lesions or infection were suspected. All samples were collected using sterile methods. Aerobic and anaerobic bacteria were collected using Fisherfinest™ Amies clear gel transport swabs (Fisher Scientific, Pittsburgh, PA). Swabs were shipped overnight to IDEXX Laboratories, Grafton, MA. Swabs sent to IDEXX were planted onto plates and incubated for 24 h. Aerobic samples were plated on blood agar and MacConkey plates, while anaerobic samples were plated on blood agar, MacConkey and anaerobic blood agar plates. Gram stains were performed on all isolates. Samples were then placed in a Vitek system (Biomerieux, Durham), which performed both bacterial identification and antibiotic susceptibility testing. The Vitek system uses biochemical testing to identify bacterial isolates, and broth microdilution and the Kirby Bauer disk method to perform antibiotic susceptibility tests according to CLSI guidelines. IDEXX used American Type Culture Collection control strains EC25922, PSA 27853, EC35218, SA 29213, EF 52199 and EF 29212. Antibiotics tested include amikacin, ampicillin, augmentin, carbenicillin, ceftazidime, ceftiofur, cephalothin, chloramphenicol, ciprofloxacin, gentamycin, tribrissen, piperacillin, enrofloxacin, tetracycline, ticarcillin and tobramycin.

Stranded and bycaught mortality samples

Marine mammals were collected with the assistance of the New England Aquarium, University of New England Marine Animal Rehabilitation Center, the National Oceanographic Atmospheric Administration (NOAA) Northeast Fisheries Science Center (NEFSC) Observer Program and the authors. Large whale cases were necropsied at the site of stranding (usually beach) while other animals were necropsied in a laboratory within 4–48 h postmortem (stored at 4 °C overnight). Full necropsies of marine mammals were conducted under protocols described by Pugliares *et al.* (2007). Stranded and bycaught birds were collected by the staff at the Seabird Ecological Assessment Network (SEANET, http://www.tufts.edu/vet/seanet/), Massachusetts Audubon Society, NOAA NEFSC Observer Program and the authors. Necropsies of marine birds were conducted using protocols as described by SEANET with tissue samples at Tufts

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University. Details of the sample source locations, species sampled, bacteria isolated and pathobiological analyses have been published (Bogomolni *et al*., 2008). Sample collection methods are summarized below briefly.

Live animal samples

Fecal samples from seals and birds were collected from beaches at the Isles of Shoals, NH/ME; Great Island in Wellfleet, MA; Muskeget Island, Nantucket Sound, MA; Monomoy National Wildlife Refuge; and Chatham Harbor, Chatham, MA. Visual identifications and photographs of the species present at each beach were made before approaching the animals and collecting feces. Animals were identified as Harbor Seal (*Phoca vitulina*), Grey Seal (*Halichoerus gryphus*), Double-crested Cormorant (*Phalacrocorax auritus*), and Herring (*Larus argentatus*) and Great Black-backed Gulls (*Larus marinus*). If $< 90\%$ of the seals at a haul-out site were a single species, fecal samples were considered as seal species 1 or seal species 2. Bacterial swabs were collected on site.

Fecal samples were collected from live-caught gulls at Appledore Island, ME, and Monomoy National Wildlife Refuge, MA. Adult Great Black-backed Gulls, Herring Gulls, and Laughing Gulls (*Larus atricilla*) were captured during egg incubation using either a walk-in nest trap (a chicken wire cage with an opening at the bottom and an entrance on one side) or a drop-down trap (chicken wire cage propped up on one side by a wooden peg attached to a line). Once a bird was trapped in either trap type, it was immediately approached and the bird gently removed and placed into a cloth cone for restraint and to prevent injury. Each bird was banded, measured and pharyngeal and cloacal swabs were collected to obtain samples of bacteria.

Data management and statistical analysis

For each bacterial isolate, information was collected about animal and tissue of origin, animal provenance (live, stranded or bycaught), location coordinates of sample collection, taxonomic identification of isolate by IDEXX and sensitivity to each of the 16 antibiotics listed above. The dataset was then manipulated to obtain a variety of general information, including the prevalence of single and multiple antibiotic resistances (MARs) across all isolates, the occurrence of antibiotic resistance within taxonomic groups of bacterial isolates, the effectiveness of each antibiotic against all bacterial isolates and the prevalence of MARs within different tissue groups across all animals. The proportion of drugs to which a particular isolate was resistant generated the MAR index (range 0–1) (Krumperman, 1983). A single MAR value was calculated for each tissue sampled in each animal by averaging MAR values for bacterial isolates from multiple swabs of the same tissue or MAR values for multiple bacteria isolated from a single swab. By averaging MAR values for multiple bacterial isolates from single tissues, we sought to minimize the potential bias of repeated sampling of single tissues and equalize the contribution of individual animals to our tissue-specific analysis. This method should also have reduced the potential bias of bacterial isolates that possess innate resistance to many antibiotics (e.g. *Chryseobacterium* and *Pseudomonas*) (Fraser & Jorgensen, 1997; Poole, 2005). Fortunately, *Chryseobacterium* and *Pseudomonas* isolates also made up a very small proportion of the dataset (20 out of 472 isolates); hence this potential bias should not have affected our analysis.

The prevalence of antibiotic resistance in bacterial isolates of different groups (birds vs. mammals; live vs. stranded vs. bycaught animals) was compared using the Storer–Kim method for comparing binomials (Storer & Kim, 1990; Wilcox, 2003). The similarity of resistance patterns across all bacterial isolates was compared for the 16 antibiotics with a cluster analysis combined with the similarity profile (SIMPROF) test using the ecological statistical software program PRIMER V6 (Clarke & Warwick, 2001; Clarke & Gorley, 2006). The Bray–Curtis coefficient was used to create a similarity matrix, and then a hierarchical agglomerative clustering method with group-average linking was used to generate a dendrogram illustrating similarities among antibiotics. The significance of clustering levels was determined using the SIMPROF test for null structure. The Bray–Curtis coefficient is particularly useful for comparing patterns of antibiotic resistance and detecting the presence of shared resistances in this study because it is independent of joint absence, in other words, similarity between two bacterial isolates is only increased if both exhibit resistance to the same antibiotic(s) (Clarke *et al*., 2006). Similarity is not affected by two isolates both exhibiting sensitivity to the same antibiotic.

The Bray–Curtis coefficient was also used to generate a similarity matrix among the subset of bacterial isolates that exhibited some antibiotic resistance. The analysis of similarity (ANOSIM) test for differences between groups of samples was used to determine the significance of similarity between antibiotic resistance profiles of bacterial isolates grouped according to animal type (birds vs. mammals) and according to animal provenance (live vs. stranded vs. bycaught organisms) (Clarke & Green, 1988). This procedure uses ranked similarities and a permutation test to compare the overall similarity of samples within groups that were created based on animal type or animal provenance to the overall similarity of samples between groups to determine the significance of differences in antibiotic resistance profiles between groups.

Results

The dataset consisted of 472 isolates of clinical interest from 149 animals tested for resistance to a suite of 16 antibiotics.

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Two hundred and eighty-seven isolates were isolated from 79 seabirds, 174 from 64 marine mammals and 11 from 6 sharks. In birds, 22 isolates were found from 5 bycaught, 109 from 34 live and 156 from 40 stranded. In marine mammals, 28 isolates were found from 12 bycaught, 56 from 31 live and 90 from 21 stranded. Fifty-eight percent of the total isolates were resistant to at least one antibiotic, while 43% were resistant to multiple antibiotics. An MAR index value ≥ 0.2 was observed in 38% of the resistant pathogens. While most isolates demonstrated resistance to at least one antibiotic, many also were resistant to multiple antibiotics (Fig. 1). The total amount of antibiotic resistance observed within individual isolates ranged from 0 to 13 antibiotics. Fourteen percent of the total isolates were resistant to a single antibiotic, 10% were resistant to two antibiotics and 33% were resistant to three or more antibiotics. Three isolates were resistant to 10 or more antibiotics.

The prevalence of antibiotic resistance within individual taxonomic groups of bacterial isolates was determined for a subset of the data. Taxa for which there were > 20 isolates were grouped and the percentage of isolates demonstrating antibiotic resistance within each of these groups was determined (Table 1). This method yielded nine total groups and a wide range of total antibiotic resistance was observed among these groups. Six of these groups had $> 70\%$ of their isolates demonstrating antibiotic resistance, which was substantially higher than the average of 57% of isolates from the whole dataset that demonstrated antibiotic resistance. However, the remaining three groups (*Edwardsiella* spp., *Escherichia coli* and a group of non-enteric gram-negative rods) had much lower antibiotic resistance on average, ranging from 14% to 30% of isolates demonstrating any antibiotic resistance.

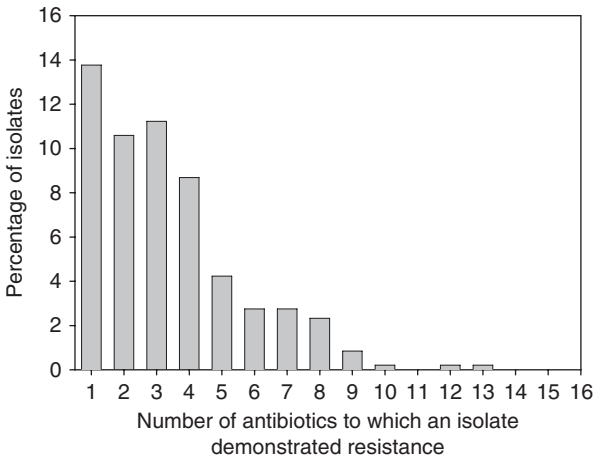

Fig. 1. Incidence of antibiotic resistance in bacterial isolates from marine mammals and seabirds. Isolates were plotted as a percentage of the total dataset based on the number of antibiotics to which they demonstrated resistance.

Table 1. Occurrence of antibiotic resistance across the nine most commonly isolated bacterial taxonomic groups

Bacterial group (total number of isolates)	Isolates sensitive to all antibiotics	Isolates resistant to one antibiotic	Isolates resistant to multiple antibiotics
Aeromonas spp. (20)	3	2	15
Edwardsiella spp. (20)	17	2	1
Enterobacter spp. (27)	2	5	20
Escherichia coli (117)	101	4	12
Non-enteric gram-negative rod (27)	19	3	5
Proteus spp. (24)	2	9	13
Shewanella spp. (43)	12	17	14
Vibrio spp. (36)	4	2	30
Vibrio alginolyticus (21)	2	1	18

Detailed information about the 18 bacterial isolates that demonstrated resistance to eight or more antibiotics is listed in Table 2. These isolates represented 10 different bacterial taxa and were isolated from marine mammals, seabirds and sharks. The provenance of these isolates included bycaught and stranded marine mammals and live and stranded seabirds. The isolates did not include any representatives from live marine mammals or bycaught seabirds. The nine tissues from which isolates were obtained also varied considerably, including both internal and external tissues.

The percentage of total bacterial isolates demonstrating resistance to each of the 16 tested antibiotics is shown in Fig. 2. The percentage of resistant isolates ranged from 1 (for ciprofloxacin and enrofloxacin) to 39 (cephalothin). Four antibiotics were ineffective against $> 25\%$ of tested isolates: carbenicillin, augmentin, ampicillin and cephalothin. Seven antibiotics were ineffective against $< 5\%$ of tested isolates: amikacin, ceftazidime, ciprofloxacin, enrofloxacin, gentamicin, tobramycin and tribrissen.

Antibiotics were clustered into groups according to similarity in resistance patterns across all tested bacterial isolates (Fig. 3). Solid lines in the cluster diagram indicate significant differences between groups of antibiotics and dotted lines with asterisks at the node indicate insignificant differences in resistance patterns between two antibiotics. The degree of similarity between two antibiotics increased only if resistance to both was observed in individual isolates. Similarity estimates were not affected by an isolate that demonstrated sensitivity to two or more antibiotics. In general, antibiotics from the same class tended to group together, i.e. aminoglycosides such as amikacin and gentamicin, penicillins such as ampicillin and carbenicillin, quinolones such as ciprofloxacin and enrofloxacin and β-lactams such as augmentin and cephalothin showed similar patterns of resistance across all bacterial isolates. However, we did observe two pairs of unrelated antibiotics with

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Table 2. Bacterial isolates demonstrating resistance to eight or more antibiotics

Animal source (common name)	Provenance	Swab location	Bacterial isolate	# Resist	Resistance profile
Harp Seal	Mammal bycatch	Thorax	*Chryseobacterium indologenes*	13	AMK, AUG, AMP, CAR, CAZ, CEF, CEPH, CHL, GEN, TET, TIC, TOB, TRI
Harbor Porpoise	Mammal bycatch	Thorax	*Sphingomonas multivorium*	12	AMK, AMP, CAR, CEF, CEPH, CHL, CIP, GEN, PIP, TET, TIC, TOB
Minke Whale	Mammal strand	Prescapular lymph	*Vibrio alginolyticus*	10	AMK, AMP, CAR, CEF, CEPH, CIP, ENR, GEN, PIP, TIC
Great Black-backed Gull	Bird strand	Coelom	*Pseudomonas* sp.	9	AUG, AMP, CAR, CAZ, CEF, CEPH, CHL, TIC, TRI
Common Dolphin	Mammal strand	Thorax	*Pseudomonas* sp.	9	AUG, AMP, CAR, CEF, CEPH, CHL, CIP, ENR, TIC
Herring Gull	Bird live	Oral	*Proteus mirabilis*	9	AMP, CAR, CEPH, CHL, GEN, PIP, TET, TIC, TOB
Hooded Seal	Mammal strand	Lymph	*Pseudomonas* sp.	9	AUG, AMP, CAR, CEF, CEPH, CHL, ENR, TET, TIC
Great Black-backed Gull	Bird strand	Coelom	*Pseudomonas* sp.	8	AUG, AMP, CAR, CEF, CEPH, CHL, TIC, TRI
Atlantic White-sided Dolphin	Mammal bycatch	Abdomen	Non-enteric gram-negative rod	8	AMK, CEF, CEPH, CIP, ENR, GEN, TET, TOB
Herring Gull	Bird live	Cloaca	Non-enteric gram-negative rod	8	AMK, AUG, AMP, CAR, CEPH, TET, TIC, TRI
Pygmy Sperm Whale	Mammal strand	Roof of Mouth	*Providencia rettgeri*	8	AUG, AMP, CAR, CEPH, CHL, PIP, TET, TIC
Pygmy Sperm Whale	Mammal strand	Oral	*Pseudomonas* sp.	8	AUG, AMP, CAR, CEF, CEPH, CHL, TET, TIC
Herring Gull	Bird live	Cloaca	*Burkholderia cepacia*	8	AUG, AMP, CAR, CAZ, CEF, CEPH, TET, TIC
Herring Gull	Bird live	Oral	*Proteus vulgaris*	8	AUG, AMP, CAR, CEF, CEPH, PIP, TET, TIC
Herring Gull	Bird live	Oral	*Pseudomonas* sp.	8	AUG, AMP, CAR, CEF, CEPH, CHL, PIP, TIC
Herring Gull	Bird live	Oral	*Proteus penneri*	8	AMP, CAR, CEPH, CHL, PIP, TET, TIC, TRI
Herring Gull	Bird live	Cloaca	*Escherichia coli*	8	AUG, AMP, CAR, CEF, CEPH, PIP, TET, TIC
Thresher Shark	Shark	Nares	*Pseudomonas* sp.	8	AUG, AMP, CAR, CEF, CEPH, CHL, TET, TIC

Details include the common name of the bird, mammal or shark from which each bacteria was isolated, the provenance of the source animal (live, stranded or bycaught), the animal tissue swabbed, the taxonomic affiliation of the bacterial isolate, the number of antibiotics to which each isolate demonstrated resistance and the resistance profile.
AMK, amikacin; AMP, ampicillin; AUG, augmentin; CAR, carbenicillin; CAZ, ceftazidime; CEF, ceftiofur; CEPH, cephalothin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; TRI, tribrissen; PIP, piperacillin; ENR, enrofloxacin; TET, tetracycline; TIC, ticarcillin; TOB, tobramycin.

similar patterns of resistance across isolates: ceftiofur (a β-lactam cephalosporin) vs. chloramphenicol and also ceftazidime (another β-lactam cephalosporin) vs. tribrissen (a sulfonamide).

Isolates were also grouped based on the marine mammal or seabird tissue from which swabs were taken (Fig. 4). Only tissues with more than five isolates were included in this analysis; this included oral, cloacal, blowhole, fecal, coelom, spleen and thorax samples. The MAR index was calculated for each of these isolates, and isolates were further grouped based on whether the MAR indices were 0, < 0.2 (amount of antibiotic resistance typical of nonpoint sources of pollution) and > 0.2 (amount of antibiotic resistance considered characteristic of point-source pollution) (Krumperman, 1983). Figure 4 illustrates the percentage of bacterial isolates within each of these three groups for each tissue. In general, tissues in contact with the environment (oral, cloaca, blowhole) had higher percentage of isolates with a MAR > 0.2 than did isolates from internal tissues (fecal,

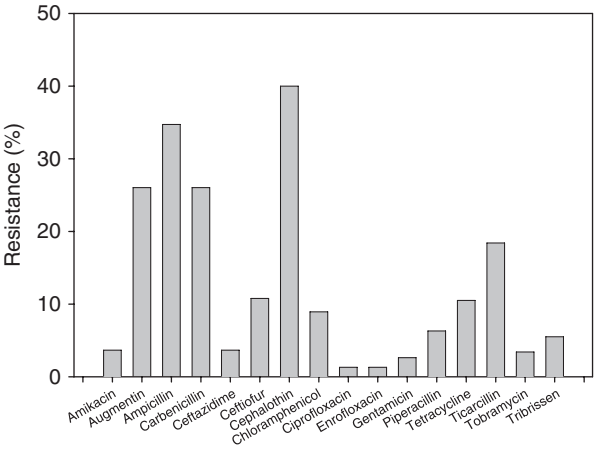

Fig. 2. Effectiveness of each antibiotic tested against the entire group of bacterial isolates. The percentage of total isolates demonstrating resistance is plotted for each antibiotic.

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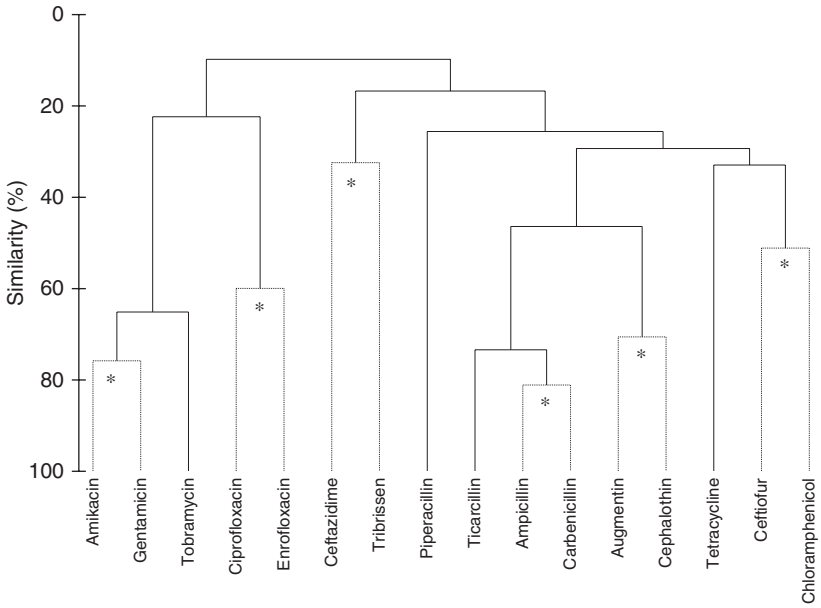

Fig. 3. Cluster dendrogram illustrating similarities among antibiotics in terms of which of the bacterial isolates demonstrated resistance. Dotted lines and asterisks indicate insignificant differences between pairs of antibiotics.

spleen, thorax). Additionally, a greater percentage of isolates from internal tissues demonstrated no antibiotic resistance than isolates from external tissues.

The ANOSIM statistical test yielded no significant groupings of resistance patterns based on either animal provenance or animal type (both $P > 0.05$). A binomial comparison of the total amount of antibiotic resistance in birds vs. mammals indicated that on average a significantly higher percentage of bacterial isolates from seabirds demonstrated resistance to at least one antibiotic than did bacterial isolates from marine mammals (61% vs. 50%; $P = 0.02$). Isolates were next subdivided into two groups based on whether they were taken from mammals or birds (the number of shark samples was too small to constitute a reasonable group). An ANOSIM test based on animal provenance yielded no significant results for seabirds ($P > 0.05$), and binomial comparisons of seabirds grouped according to animal provenance yielded no significant differences in the occurrence of antibiotic resistance among bacterial isolates from live, stranded or bycaught birds (all $P > 0.05$). An ANOSIM test based on animal provenance was significant for marine mammals; however ($P = 0.02$), indicating significant differences in antibiotic resistance patterns between live and bycaught marine mammals ($P = 0.04$) and between live and stranded marine mammals ($P = 0.02$), but no significant differences in resistance patterns between stranded and bycaught marine mammals ($P = 0.09$). These results indicate that live mammals as a group were resistant to different types of antibiotics than were stranded and bycaught marine mammals. Additionally, a binomial comparison of live vs. bycaught or stranded marine mammals indicated that the percentage of bacterial isolates demonstrating antibiotic resistance from live mammals was significantly lower than

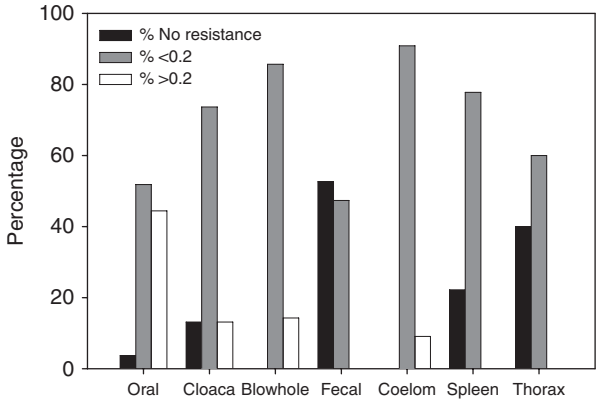

Fig. 4. Incidence of MARs among bacteria isolated from different animal tissues. Isolates were grouped into three categories according to whether they were sensitive to all antibiotics MAR index = 0, MAR < 0.2 but > 0 or MAR > 0.2. The percentage of isolates in each category is plotted for each tissue.

the isolates from bycaught or stranded marine mammals ($P < 0.001$ for both comparisons). The percentage of antibiotic-resistant bacterial isolates from bycaught marine mammals was not significantly different than that observed in stranded marine mammals ($P = 0.56$).

Discussion

Comparison to other studies of antibiotic resistance in terrestrial and aquatic animals

Our study contains one of the largest and most diverse datasets in terms of both the variety of marine animals and

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tissues sampled and the bacterial groups isolated. In general, most studies of bacteria isolated from mammals and birds have reported relatively high prevalence of antibiotic resistance and similar patterns of effectiveness across antibiotics as those observed here. In bacteria isolated from stranded Harbor Seals over a 12-year period, Lockwood *et al.* (2006) observed widespread antibiotic resistance, with only one antibiotic capable of killing or inhibiting growth of all isolates tested. The authors did not report results for overall percentage of isolates resistant to one or multiple antibiotics, but did observe similar patterns in antibiotic-specific results to ours, with cultures exhibiting resistance most frequently to ampicillin (74% resistant) and cephalothin (64% resistant). A study of sharks from a variety of locations, including waters off Belize, Florida, coastal and offshore Louisiana and Massachusetts, found a high prevalence of antibiotic resistance in bacteria isolated from cloacal swabs (75%, 86.5%, 62%, 52% and 87.5% for the five locations, respectively) (Blackburn, 2003). Dolejska *et al.* (2007) reported lower total occurrence of antibiotic resistance in isolates from Black-headed Gulls (30% vs. 58% observed in this study), but all of their isolates were *E. coli* and in our study this taxon had the lowest antibiotic resistance when compared with other bacterial groups (14% of our *E. coli* were susceptible to all antibiotics vs. 58% of all isolates tested). Another study of seabirds from rehabilitation centers on the Pacific coast of the United States had a much smaller sample size (19 isolates from 15 birds) but saw high occurrence of antibiotic resistance, with 68% of isolates demonstrating resistance to at least one antibiotic (Steele *et al.*, 2005). These authors reported high levels of resistance to ampicillin, augmentin and cephalothin, which was consistent with our results, but also observed a high degree of resistance to ceftiofur (37% resistant), which was relatively effective against our bacterial isolates (10% resistant). Even higher levels of antibiotic resistance were reported by Bass *et al.* (1999), in a study of antibiotic resistance in *E. coli* isolates from diseased poultry at the Poultry Diagnostic and Research Center, University of Georgia. Virtually all isolates were resistant to at least one antibiotic, and 64% were resistant to five or more antibiotics. In contrast, a study of bacterial isolates from zoo animals in Japan found that 21% of isolates tested for resistance to a wide spectrum of antibiotics demonstrated resistance to two or more antibiotics, which is approximately half the occurrence of multiply resistant isolates we observed here (43%) (Ahmed *et al.*, 2007). Overall, reports on ABR bacteria from animals, and marine animals in particular, indicate not only the widespread presence of these microorganisms, but often a significant percentage of the bacteria demonstrating resistance to multiple antibiotics.

Antibiotic resistance in our samples

We observed widespread occurrence of antibiotic resistance and MAR in our samples (Fig. 1), which was consistent with many of the studies discussed in the previous section. An MAR index value ≥ 0.2 was observed in 38% of the resistant pathogens, suggesting exposure of the animals to bacteria from significantly contaminated sites. High MAR index values have been shown to be indicative of environments with high enteric disease potential (Krumperman, 1983). However, we noted that this high occurrence of resistance was not evenly distributed across all antibiotics, bacterial taxonomic groups or all tissues sampled. The 16 antibiotics tested showed a wide range of effectiveness against the bacterial isolates, from 99% effective (ciprofloxacin and enrofloxacin) to 61% effective (ampicillin). The four least effective antibiotics were ineffective against $> 25\%$ of tested isolates and included cephalothin, ampicillin, augmentin and carbenicillin. Relatively high occurrence of antibiotic resistance against ampicillin, augmentin and cephalothin has been reported previously in environmental isolates (Boon & Cattanach, 1999; Miranda & Zemelman, 2001; Steele *et al.*, 2005; Lockwood *et al.*, 2006; Dolejska *et al.*, 2007; Lima-Bittencourt *et al.*, 2007; Watkinson *et al.*, 2007), although this is not always the case (Bass *et al.*, 1999; Edge & Hill, 2005). To our knowledge, high occurrence of resistance to carbenicillin has not been reported previously. While bacterial resistance to ciprofloxacin, enrofloxacin and ceftazidime was low within the overall group, the demonstration of resistance by some isolates to these front-line antibiotics is noteworthy for its illustration of the diversity and widespread nature of antibiotic resistance within our environmental samples.

Among groups of bacterial isolates commonly sampled, occurrence of antibiotic resistance ranged from 14% of isolates (*E. coli*) to 92% (*Enterobacter* spp.) (Table 1). We were surprised to see such a range of resistance across different taxonomic groups, in particular to see such low occurrence of antibiotic resistance in *E. coli* when compared with other taxonomic groups and to the overall average for the dataset as a whole. *Escherichia coli* is often used exclusively to determine occurrence of antibiotic resistance in an environment in general, and for source tracking of fecal pollution based on antibiotic resistance patterns (e.g. Krumperman, 1983; Parveen *et al.*, 1997; Bass *et al.*, 1999; Kelsey *et al.*, 2003; Edge & Hill, 2005; Dolejska *et al.*, 2007; Kaneene *et al.*, 2007; Sjolund *et al.*, 2008). Our results are consistent, however, with two studies examining antibiotic resistance across a variety of bacterial taxa. Boon & Cattanach (1999) compared antibiotic resistance in *E. coli* and native heterotrophic bacteria isolated from the Yarra River, Australia. This study reported significantly greater incidence of antibiotic resistance in native heterotrophic bacteria than in *E. coli* isolated from the same sites. Lima-Bittencourt *et al.* (2007) also reported much lower incidence of MAR in *E. coli* isolates relative to nine other enterobacterial taxa. These results highlight the importance of examining a range of

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bacteria in order to determine an accurate representation of antibiotic resistance in an environment.

We did not observe any consistent trends in the group of isolates resistant to the largest number of antibiotics (Table 2). The isolates spanned a wide range of animal types, animal provenance, tissue types and bacterial taxonomic groupings. We did observe differences in occurrence of MARs across animal tissues sampled (Fig. 4). Most studies of bacteria isolated from animals have used swabs of single tissues or feces to characterize the incidence of single and multiple antibiotic resistances in the animal as a whole. Our results are in contrast, however, with one study that found no differences in the incidence of antibiotic resistance between bacteria isolated from swabs of gills and intestinal content in pelagic and demersal fish (Miranda & Zemelman, 2001). In our study, tissues that came into direct contact with the environment (oral, cloacal, blowhole) had higher incidence of bacteria with resistance to multiple antibiotics, and lower incidence of no resistance, than internal tissues (spleen, thorax) and fecal samples. Most of the bacteria isolated from fecal samples were *E. coli*, which had a relatively low incidence of antibiotic resistance compared with other bacterial taxonomic groups. It is not possible in our dataset to determine whether it was the sample type (feces), bacterial group, or both, that had low occurrence of antibiotic resistance. However, bacteria isolated from the spleen and thorax belonged to a range of different taxonomic groups, suggesting results for these two internal tissues were tissue specific rather than bacterial group specific (data not shown). The animals themselves may not be harboring large internal pools of antibiotic-resistant bacteria. High levels of antibiotic resistance have been reported to occur in biofilms (Stewart & Costerton, 2001; Gilbert *et al.*, 2002). It may be possible that the oral, cloacal and blowhole swabs sampled biofilms that are commonly present on external tissues.

Implications for animal health

The occurrence of antibiotic resistance was higher in bacteria isolated from seabirds than from marine mammals. Within marine mammals, there were also significant differences between the occurrence of antibiotic resistance in live vs. stranded and live vs. bycaught animals. Additionally, there were differences in the patterns of antibiotic resistance, or the groups of antibiotics to which bacteria demonstrated resistance, between live vs. stranded and live vs. bycaught animals. We did not observe these differences among live, stranded or bycaught seabirds in either the incidence of antibiotic resistance or the patterns of antibiotic resistance. The differences between marine mammals and seabirds may be due to different diet and habitat. Seabirds live and forage largely in nearshore coastal environments, which may result in increased exposure to either highly impacted sites (sewage treatment ponds, landfills) or bacteria brought to the marine environment in runoff from highly impacted sites (Nelson *et al.*, 2008). These results may also indicate that live marine mammals are generally healthier than their stranded or bycaught counterparts. It has been a tacit assumption that bycaught animals represent a subsample of the 'healthy' population. This needs further examination given our findings in this study. A confounding factor in the analysis is that most of the samples from live animals were fecal swabs, and as described above, most of the bacteria isolated from the fecal swabs were *E. coli*. Both fecal samples and *E. coli* showed relatively low incidence of antibiotic resistance relative to other tissues and other bacterial groups. Our dataset unfortunately does not contain a significant number of non-live-mammal fecal or *E. coli* samples. Thus, it is possible that the differences between the live vs. stranded and live vs. bycaught mammals in terms of both incidence of ABR bacteria and patterns of antibiotic resistance may have been due to the sample types collected.

Implications for antibiotic resistance in the environment

We observed large variability in the incidence of antibiotic resistance among taxonomic groups of bacterial isolates (Table 1). These results indicate the need for expanding the scope of studies that seek to characterize antibiotic resistance in an environment based on a single indicator organism. This needed expansion of common current methodology was also suggested by the variability in MARs observed in different tissues sampled from the marine mammals and seabirds examined in this study. It may be that accurate characterization of antibiotic resistance in bacterial isolates from animal hosts should include multiple swabs from a range of tissues when possible.

This study examined the occurrence of antibiotic resistance in bacteria isolated from marine mammals and seabirds, and did not include samples from the coastal marine environment itself. The results of the tissue-specific MAR analysis suggested the possibility for relatively high incidence of antibiotic resistance in the surrounding environment. We do not, however, have direct evidence that the origin of antibiotic resistance in our samples was the coastal environment itself. There have been a few studies that compared the occurrence of antibiotic resistance in both animals and their surrounding environment, with mixed results. Edge & Hill (2005) reported slightly higher incidence of MAR in fecal swabs from seabirds (average MAR, 0.07) than samples of surface waters (average MAR, 0.059). Parveen *et al.* (1997) reported much lower incidence of antibiotic resistance among fecal samples from terrestrial wild animals (27.6% of bacterial isolates resistant to at least

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one antibiotic) than from local estuarine surface waters (82% resistant isolates). However, Watkinson *et al.* (2007) found that the incidence of antibiotic resistance among bacterial isolates from oysters exposed to wastewater treatment plant discharge was much lower than from the discharge itself. Resistant isolates were found in 4% of the oysters vs. 31% of the discharge samples. Based on these discrepancies, it is thus unclear whether the high levels of antibiotic resistance observed are reflective of the larger coastal environment.

Conclusions

In summary, we observed widespread antibiotic resistance in bacterial isolates from a range of marine mammals and seabirds, and the high incidence of single and multiple antibiotic resistances in this study was consistent with other studies of bacterial isolates of animal origin. The source of antibiotic resistance in bacterial isolates from these marine mammals and seabirds is not clear. Some of these animals live in nearshore waters and/or come into regular contact with human populations, but MAR was also observed in marine mammals that inhabit offshore, deep water far from the presumed impact of coastal human populations. However, the widespread occurrence of single and multiple antibiotic-resistant bacterial isolates from these marine mammals, as well as the relatively high occurrence of antibiotic resistance on external tissues sampled may reflect a large environmental pool of ABR bacteria in coastal waters of the Northeastern United States. We found large variability in the occurrence of antibiotic resistance both across bacterial taxonomic groups and animal tissues sampled, highlighting the potential need for the expansion of current common practices of single tissue samples and single indicator organisms to assess the incidence of antibiotic resistance in an animal or the environment. On sampling within the group of animals, the observed differences in incidence of antibiotic resistance between marine mammals and seabirds may be caused by differences in behavior and lifestyle and reflect the greater general exposure of seabirds to sources of human pollution. Additionally, the differences in both incidence and patterns of antibiotic resistance among live, stranded and bycaught marine mammals may be indicative of differences in overall animal health.

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