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MOLECULAR DETECTION OF ANTIBIOTIC-RESISTANCE DETERMINANTS IN *ESCHERICHIA COLI* ISOLATED FROM THE ENDANGERED AUSTRALIAN SEA LION (*NEOPHOCA CINEREA*)

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ABSTRACT: Greater interaction between humans and wildlife populations poses significant risks of anthropogenic impact to natural ecosystems, especially in the marine environment. Understanding the spread of microorganisms at the marine interface is therefore important if we are to mitigate adverse effects on marine wildlife. We investigated the establishment of *Escherichia coli* in the endangered Australian sea lion (*Neophoca cinerea*) by comparing fecal isolation from wild and captive sea lion populations. Fecal samples were collected from wild colonies March 2009–September 2010 and from captive individuals March 2011–May 2013. Using molecular screening, we assigned a phylotype to *E. coli* isolates and determined the presence of integrons, mobile genetic elements that capture gene cassettes conferring resistance to antimicrobial agents common in fecal coliforms. Group *B2* was the most abundant phylotype in all *E. coli* isolates ($n=37$), with groups *A*, *B1*, and *D* also identified. Integrons were not observed in *E. coli* ($n=21$) isolated from wild sea lions, but were identified in *E. coli* from captive animals ($n=16$), from which class I integrases were detected in eight isolates. Sequencing of gene cassette arrays identified genes conferring resistance to streptomycin-spectinomycin (*aadA1*) and trimethoprim (*dfrA17*, *dfrB4*). Class II integrases were not detected in the *E. coli* isolates. The frequent detection in captive sea lions of *E. coli* with resistance genes commonly identified in human clinical cases suggests that conditions experienced in captivity may contribute to establishment. Identification of antibiotic resistance in the microbiota of Australian sea lions provides crucial information for disease management. Our data will inform conservation management strategies and provide a mechanism to monitor microorganism dissemination to sensitive pinniped populations.

Key words: Captivity, integron (*intI1* and *intI2*), phylotyping, pinniped, wildlife.

INTRODUCTION

Interactions at the human–domestic animal–wildlife interface have facilitated the movement of microorganisms from terrestrial sources to marine ecosystems (Halpern et al. 2008). In particular, antibiotic-resistant bacteria are becoming more common in marine wildlife populations (Stoddard et al. 2008; Rose et al. 2009), indicating colonization by terrestrial bacteria or integration of antibiotic-resistance determinants in the microbiota of wildlife species. The mobility of DNA elements encoding antibiotic resistance has been instrumental in the rapid spread of antibiotic resistance, both in a clinical setting and in naturally occurring ecosystems (Partridge et al. 2009). Mobile DNA elements (integrons) have been particularly important in emergence of antibiotic resistance. Integrons encode for integrase genes (*int*) that enable insertion of gene cassettes at a recombination site (*attI*) and subsequent expression at an adjacent promotor (P_c) (Hall and Collis 1995; Collis et al. 1998). Integrons can be classified into classes I, II, and III, with class I being most common in clinical pathogens (Stokes and Hall 1989). Class I integrons integrate within transposons or plasmids, a feature that has further facilitated their spread within and between species of bacteria (Gillings et al. 2008).

The presence of antimicrobial-resistance determinants in coastal seawater and wastewater runoff is well documented (e.g., Reinthaler et al. 2003; Schwartz et al. 2003). Exposure of wildlife to untreated wastewater may present a pathway for transfer of human-derived bacteria, and the antibiotic resistance genes they carry,

to wildlife populations (Pellegrini et al. 2009). Ultimately, this may lead to colonization by microorganisms atypical of the natural habitats of wildlife species (Power et al. 2013). Class I integrons reported in clinical pathogens are commonly identified in aquatic vertebrates, including gulls, flamingo, carp, salmon, and catfish, all animals frequently exposed to anthropogenic impact in their natural habitats (e.g., McIntosh et al. 2008; Dolejska et al. 2009).

Culture-based screening and antibiotic sensitivity testing have identified resistance to greater than 10 antibiotics across six seal species (e.g., Lockwood et al. 2006; Rose et al. 2009). However, these techniques are unable to identify specific resistance determinants and their origins, and this knowledge gap limits understanding of dispersal of antibiotic resistance in wild marine populations (Stokes et al. 2001).

The Australian sea lion (*Neophoca cinerea*) is an endangered marine mammal endemic to Australia. The total population is estimated at <15,000 animals; the only site monitored in the long-term, Kangaroo Island, shows continual decline (Shaughnessy et al. 2011). Australian sea lions breed in at least 76 small, dispersed colonies on islands and some protected coves from Houtman Abrolhos in Western Australia (WA) to The Pages in South Australia (SA) (Shaughnessy et al. 2011). Their geographic range stretches across >2,700 km of coastline and encompasses two major cities of >1 million people (Perth, WA, and Adelaide, SA) and a number of large rural centers, as well as some very isolated sites. Although their distribution is broad, individual colonies show limited dispersal, localized foraging by both sexes, and a high degree of population genetic structure (Lowther et al. 2012, 2013). Sea lions are indirectly exposed to terrestrial microorganisms through ingestion of seawater contaminated with wastewater runoff and via behaviors such as hauling-out on beaches used by humans (Gales et al. 1994). In addition, as a tourist icon, sea lion colonies in both WA and SA receive high levels of human visitation, with visitor numbers to Seal Bay on Kangaroo Island exceeding 150,000 tourists annually (Goldsworthy et al. 2007). Such interactions significantly increase the risk of introducing atypical microbes to vulnerable wildlife populations (Daszak et al. 2000; Skurnik et al. 2006). We hypothesized that sea lions in captive environments and wild colonies exposed to increased anthropogenic impact may have higher levels of *Escherichia coli* carrying class I or class II integrons than isolated sea lion populations with limited disturbance. We screened isolates of *E. coli* from sea lion populations using PCR for class I and class II integrons. We also examined the relationship between *E. coli* presence and phylotype distribution.

MATERIALS AND METHODS

Fecal sample collection

Australian sea lion fecal samples ($n=271$) were collected over 2 yr from 11 coastal and island colonies in SA and WA (Table 1). Fecal samples were also collected from captive animals ($n=19$) at Dolphin Marine Magic and Taronga Zoo, New South Wales, and Sea World, Queensland, over 4 mo. Fecal samples were transported to the laboratory and stored at 4 C until processed.

Enrichment for *E. coli*

To obtain pure cultures of *E. coli*, Chromocult agar plates (Merck, Darmstadt, Germany) were streak-inoculated with feces using sterile swap applicators and incubated at 37 C for 24 h (Finney et al. 2003). Following the absence of *E. coli* isolation from agar plates, MacConkey enrichment broth (2 mL; Oxoid, Hampshire, UK) was inoculated with fecal material (approximately 500 mg), and broth cultures were incubated at 37 C for 24 h. Enriched broth cultures were streak-inoculated onto Chromocult agar plates. Colonies positive for *E. coli*, as indicated by dark-blue to violet growth, were selected to establish pure cultures.

DNA extraction and PCR protocols

Genomic DNA was extracted from *E. coli* cultures using the Isolate Fecal DNA Kit (Bioline, Sydney, Australia) according to

manufacturer's protocol. We determined PCR competency of extracted DNA by 16S rRNA amplification using the universal eubacterial primers *F27* and *R1492* (Yeates and Gillings 1998). Reactions were performed using the DNA polymerase GoTaq® Green 2X Master Mix (Promega, Madison, Wisconsin, USA).

To assign *E. coli* isolates to a phylotype, DNA extracted from *E. coli* cultures ($n=37$) was amplified according to Clermont et al. (2000) using GoTaq Green Master Mix (Promega). Phylotype groups were determined by the presence and absence of fragments associated with each of the *ChuA* (279 base pairs [bp]), *YjaA* (211 bp), and *TSP* (152 bp) primer pairs using gel electrophoresis (2% w/v, 110 V for 30 min) in TBE (Tris, boric acid, ethylenediaminetetraacetic acid, pH 8) with SYBR safe (Invitrogen, Mulgrave, Australia). Product size was approximated against a HyperLadderII DNA marker (Bioline, Sydney, Australia). Positive controls representing *E. coli* phylotypes *A, B1, B2,* and *D* were used in all reactions (Power et al. 2005).

To screen for class I integrons, an integrase (*intI1*)–specific PCR was performed using the primers *HS463a* and *HS464* (Gillings et al. 2008). *HS463a* and *HS464* amplify an internal fragment of the class I integron integrase (*intI1*) gene producing an ~473-bp product. DNA polymerase GoTaq Colourless Master Mix (Promega) was used for amplification, and cycling consisted of initial denaturation for 3 min at 94 C; 35 cycles at 94 C for 30 s, 60 C for 30 s, and 72 C for 1 min 30 s; and a final extension step at 72 C for 5 min. A spiked analysis using DNA extracted from a class I integron–positive laboratory strain was performed on negative samples.

Samples producing *intI1* amplicons were further amplified to determine gene cassette arrays using the primers *HS458* and *HS459*, which amplify from the *attI1* region and the 3′ conserved segment, a region spanning the gene cassette array (Stokes et al. 2006). PCR cycling conditions included initial denaturation at 94 C for 3 min; 35 cycles at 94 C for 30 s, 60 C for 30 s, and 72 C for 1 min 30 s; and a final extension step at 72 C for 5 min. Amplicons will vary in size, depending on the number and types of gene cassettes that are present. The *intI1*-positive samples that failed to amplify using *HS458* and *HS459* were screened using the primers *MRG284* and *MRG285* (Gillings et al. 2009) using PCR conditions as described for *HS458* and *HS459* primers except for the annealing temperature and time, which were increased to 65 C for 2 min.

To screen for class II integrons, the integrase-specific PCR for integrase 2 (*intI2*) was performed using the primers *IntI2F* and *IntI2R* (Mazel et al. 2000). *IntI2F* and *IntI2R* amplify an internal fragment of the class II integron integrase (*intI2*) gene. PCR was performed using GoTaq Colourless Master Mix (Promega), and PCR cycling was done as described above for *intI1* amplification. All reactions were resolved using agarose gel electrophoresis as described already.

DNA sequencing and analyses

Amplicons from *HS463a/HS464* and *HS458/HS459* were purified for sequencing using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia). For cassette array determination, overlapping sequence fragments were obtained using the primers *HS458*, *HS459*, and *HS320* (Murray et al. 1988). All sequencing was performed by Macrogen Inc. (Seoul, Korea) on a 3130x1 genetic analyzer (Applied Biosystems, Foster City, California, USA) using the standard run protocol for a 50-cm, 16-capillary array using a Big Dye terminator kit (Applied Biosystems). Sequences generated from *HS458*, *HS320*, and *HS459* were assembled using GeneiousPRO version 5.4.6 (Biomatters Ltd., Auckland, New Zealand), and a consensus sequence was extracted. Sequences were annotated by hand after performing BlastN and BlastX searches (NCBI 2014), open reading frames were identified using BlastX comparisons, and the core sequences (GTTRRRY) and recombination site (*attC*) were used to identify gene cassette arrays. GenBank flat files were generated for submission to GenBank using BankIt. Sequences generated from this study are lodged as GenBank accession numbers KP314737–KP314740.

Statistical analyses

Fisher's exact test was used to test for differences between wild and captive populations in the prevalence and phylotype distribution of *E. coli* isolates, using the SISA Fisher's exact test calculator for up to 2×5 contingency tables (SISA 2014).

RESULTS

Culture and phylotyping

Isolation of *E. coli* using coliform selective media resulted in low yields, with isolation from 21 wild sea lion fecal

TABLE 1. *Escherichia coli* isolate yields and phylogenetic groupings from Australian sea lion (*Neophoca cinerea*) colonies and captive facilities. *E. coli* isolates were cultured from 21 wild and 16 captive sea lions. Low yields of *E. coli* were recovered from wild sea lions compared to captive individuals. Isolates from *E. coli* phylotype *B2* were most commonly recovered in samples from both wild and captive animals. Phylogenetic groups *B1*, *D*, and *A* were the next most abundant in similar distribution pattern.

Colony	Geographic coordinates[a]	Sample size (no. samples)	*E. coli* isolates (no. cultured)	*E. coli* phylotype[b]	Gene cassette array
South Australia					
Blefuscu Island	32°28′15″S, 133°37′39″E	37	6	*A* (1), *B2* (5)	
Cape Gantheaume	36°00′37″S, 137°28′28″E	7	0	NA	
Lewis Island	34°57′25″S, 136°01′54″E	21	1	*B2* (1)	
Liguanea Island	34°59′54″S, 135°37′11″E	24	0	NA	
Lilliput Island	32°28′01″S, 133°38′38″E	38	3	*B2* (2), *D* (1)	
Olive Island	32°43′25″S, 133°57′53″E	62	5	*B1* (2), *B2* (2), *D* (1)	
Seal Bay	35°59′55″S, 137°21′45″E	38	3	*B1* (1), *B2* (2)	
Seal Slide	36°03′43″S, 137°29′36″E	5	1	*B2* (1)	
West Waldegrave Island	33°35′46″S, 134°45′41″E	9	0	NA	
Western Australia					
Beagle Island	29°48′23″S, 114°52′37″E	15	1	*B2* (1)	
North Fisherman Island	30°07′47″S, 114°56′38″E	15	1	*D* (1)	
Captive					
Dolphin Marine Magic		12	10	*A* (2)	
				B2 (7)	|*aadA1*|*qacEΔ*| (1), |*dfrB4*|*qacEΔ*| (2)
				D (1)	
Sea World		5	3	*A* (1)	
				B1 (2)	*aadA*|*dfrA17* (1)
				B2 (1)	|*qacEΔ*| (1)
Taronga Zoo		2	2	*B2* (1), *D* (1)	

[a] Geographic coordinates were determined using the Google Earth satellite mapping database (Google 2010).

[b] Phylogenetic groups were determined by multilocus enzyme electrophoresis; NA = not applicable.

samples (7.7%) compared to 16 captive individuals (84%; Table 1). The yield of *E. coli* from captive and wild animals was significantly different (Fisher's exact test, two-sided $P=<0.001$). A low recovery of *E. coli* was reflected in initial direct streak plate isolation; however, subsequent enrichment using MacConkey broth and Chromocult agar increased coliform recovery rates by 34%.

In both captive and wild sea lion populations, the four phylotypes of *E. coli* isolates were not evenly distributed. The phylotype group distribution pattern was not significantly different between the two populations (Fisher's exact test, two-sided $P=0.590$). In wild animal fecal samples, phylotype *B2* was the most common (67% of isolates; Table 1), followed by phylotypes *B1* and *D* (14%

each), with phylotype *A* represented by one *E. coli* isolate (5%). Phylotype frequency was similar for captive animal fecal samples, with phylotype *B2* represented by nine isolates (56%), phylotype *A* by three isolates (19%), and phylotypes *B1* and *D* represented by two isolates (13% each).

Detection and characterization of class I and class II integrons

Genomic DNA was successfully extracted from the 37 *E. coli* isolates, and DNA from all isolates was PCR competent by *16S rRNA* screening. Class I integrase screening (*intI1*) of DNA samples resulted in no amplicons in *E. coli* DNA from wild animals ($n=21$). We detected *intI1* amplicons in eight captive animal samples (Table 1). Class II integrons were not detected in DNA samples from wild or captive sea lion populations. All DNA samples were positive when spiked for class I and class II integron screening, confirming PCR competency for these targets. DNA sequencing and BlastN searches confirmed that *intI1* amplicons ($n=8$) represented class I integrase.

Gene cassette arrays were amplified in seven of the eight *intI1* positive isolates. Successful DNA sequencing identified four constructs: |*aadA1*|*qacEΔ*| in one sample (MQ-DMM3), |*dfrB4*|*qacEΔ*| in two samples (MQ-DMM5 and MQ-DMM10), and an empty cassette |*qacEΔ*| in one sample (MQ-S5). A mixed cassette array containing partial *dfrA17* and *aadA* genes was identified in one sample (MQ-S4). The *aadA1*, *dfrB4*, and empty gene cassette constructs were identified in *E. coli* strains phylotyped as *B2*, while the mixed gene cassette array was identified in a strain phylotyped as *B1*.

DISCUSSION

The significantly lower prevalence of *E. coli* in wild Australian sea lions compared to captive animals suggests that *E. coli* may not be a dominant member of natural gut microbial communities. The low occurrence of *E. coli* in wild Australian sea lions is similar to findings from other pinniped populations, including harbor seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*), and northern elephant seals (*Mirounga angustirostris*), where *E. coli* isolates were undetected in isolated fecal coliforms (Johnson et al. 1998). In contrast, *E. coli* was frequently identified in captive Australian sea lions, indicating that the increased presence may be influenced by conditions experienced in captivity. Dissimilarities in gut microbiota abundance and richness between wild and captive animals have been observed in leopard seals (*Hydrurga leptonyx*), where variation was attributed to dietary changes experienced in captivity (Nelson et al. 2013). Captive Australian sea lions are fed fish with a limited range in terms of both species and size that have been processed through fish markets, while wild sea lions have a broad diet of primarily benthic prey, including octopus (*Octopus* spp.), giant cuttlefish (*Sepia apama*), squid (*Sepioteuthis australis*), and southern rock lobster (*Jasus edwardsii*) from inshore to the shelf edge (McIntosh et al. 2007; Gibbs et al. 2011). The introduction of microbes from unnatural prey may be contributing to variations in microbial diversity in captivity. However, as the core gut microbial community structure of the Australian sea lion remains undefined, further molecular characterization is required to infer the impact of captivity on microbial prevalence.

Identification of *E. coli* phylotypes prominent in Australian sea lions has revealed the potential for *E. coli* harboring antibiotic-resistance determinants to penetrate the animals gut biota. Of the four *E. coli* phylotype groups (*A*, *B1*, *B2*, *D*), phylotype *B2* has the highest presence of extra-intestinal virulence factors and has been suggested to have the greatest resistance to antibiotics (e.g., Johnson et al. 2001; Skurnik et al. 2005). Typically phylotype *B2* is dominant in *E. coli* strains

isolated from human and omnivorous terrestrial Australian mammals, while *B1* is the most abundant phylotype in carnivorous species (Gordon and Cowling 2003; Escobar-Páramo et al. 2006). In the case of the Australian sea lion, *B2* is the most abundant phylotype, representing 67% of isolates from wild populations and 56% in captive individuals. The dominance of phylotype *B2* may increase the risk of transfer of antibiotic-resistance genes from the surrounding environment to endangered species and warrants further investigation. Further genetic characterization assessing *B2* phylotype strain variation and virulence carriage in sea lion isolates would provide insight into the potential for establishment of antimicrobial resistance genes in wild sea lions.

Class I integrons containing diverse gene cassettes were detected in *E. coli* strains identified as *B1* and *B2* from captive sea lions. Class II integrons were not detected in either wild or captive populations. One of the genes present in the class I integrons (*aadA1*) is commonly found in both environmental and human clinical strains and encodes resistance to streptomycin-spectinomycin (Partridge et al. 2009). The second and third genes (*dfrA17* and *dfrB4*) identified in *E. coli* from captive sea lions confer resistance to trimethoprim (Partridge et al. 2009). The presence of class I integrons with gene cassette arrays similar to those commonly found in human clinical cases provides a useful indicator of potential microbial flow through the captive environment. We only found integrons in captive animals, indicating that the presence of resistance genes in *E. coli* from captive sea lions may result from conditions experienced in captivity and the dissemination of human-derived microbes at this interface. Similar findings have been reported in northern elephant seals introduced to rehabilitation centers, where time elapsed in captivity and veterinary treatment significantly increased resistance profiles of gastrointestinal *E. coli* (Stoddard et al. 2009). These findings suggest that captive environments play a major role in the establishment of antimicrobial-resistance genes (Sidjabat et al. 2006; Skurnik et al. 2006). Future observation including molecular characterization of samples from the animal housing and enclosure water may provide useful insight into factors driving selection of antibiotic-resistance genes in gut microbiota.

Use of antimicrobial agents in companion animal and wildlife veterinary care may also contribute to increasing the proportion of enteric bacteria harboring antibiotic-resistance determinants (Smith et al. 2002; Sidjabat et al. 2006), presenting two specific issues. First, persistent use of antibiotics may limit the ability to treat bacterial infection and increase the incidence of infectious disease in marine mammals (McEwen and Fedorka-Cray 2002; Allen et al. 2010). Second, selection for antibiotic-resistant bacteria provides a reservoir of resistance that may potentially be transferred to microbiota of other captive animals (Stoddard et al. 2009). Greater understanding of microbial flow through captive environments is therefore essential for determining the potential for antibiotic-resistance gene dissemination in wildlife.

While we did not detect resistance determinants in wild populations, the potential risk for antimicrobial movement to sea lion colonies exists and is clearly possible, given our findings in captive sea lions. Although most Australian sea lion colonies are found in isolated areas or on coastal islands where unlicensed visits are prohibited, three South Australian colonies, Seal Bay, Seal Slide, and Cape Gantheaume, all on Kangaroo Island, experience high levels of human tourist activity and visitation (Gales et al. 1994; Shaughnessy 1999). Several haul-outs (nonbreeding sites) in WA are also subject to very high levels of tourist visitation (Orsini et al. 2006). The high frequency of such interactions within the natural

habitat of sea lions increases the potential risk for exposure to foreign microorganisms and consequent colonization in sea lion coliforms (Skurnik et al. 2006; Allen et al. 2010).

The human–domestic animal–wildlife interface represents an area of emerging disease, zoonoses, and public health concern. For endangered endemics such as the Australian sea lion, understanding the ecology of their microbiota will provide insight into microbial dissemination routes. Given the vulnerability of pinnipeds and other colonially breeding animals to high rates of pathogen transmission, this is an area of research requiring further pursuit (Härkönen et al. 2006; Lynch et al. 2011). If we are to mitigate the effects of adverse microbial transfer, we must first identify mechanisms of dispersal of atypical microbes. The methods outlined in this paper are a first step along this path.

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