The effect of rehabilitation of northern elephant seals (*Mirounga angustirostris*) on antimicrobial resistance of commensal *Escherichia coli*

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ABSTRACT

The aim of this study was to determine if antimicrobial drug use increases resistance of commensal gastrointensinal *Escherichia coli* of wild northern elephant seals (*Mirounga angustirostris*) treated in rehabilitation, and, if so, identify the risk factors involved. Minimum inhibitory concentration (MIC) levels of twelve antimicrobial drugs were determined for 289 *E. coli* isolates from 99 seals sampled at admission and 277 isolates obtained at release from rehabilitation using broth microdilution. Prevalence of *E. coli* antimicrobial resistance, MIC_{50}, MIC_{90}, and clustering of MIC values were determined for seals and the data were analyzed using Fisher's exact test, ordinal logistic regression and negative binomial regression. At release from rehabilitation 77.8% of the seals had antimicrobial resistant *E. coli* compared to 38.4% of the seals at admission. The MIC_{90} for amoxicillin-clavulanic acid, chloramphenicol, enrofloxacin, ticarcillin-clavulanic acid, and trimethoprim-saulfamethoxazole were at levels considered to be sensitive at admission but they increased to levels of resistance at release. *E. coli* were grouped into four clusters by their MIC values, with increasing levels of resistance going from Cluster 1 to 4. A primary risk factor associated with the probability of a seal having *E. coli* in Clusters 3 and 4 was time in rehabilitation, regardless of whether the animal received treatment with antimicrobial drugs, suggesting nosocomial infection. The results of this study provide evidence that increased levels of hygiene and appropriate use of antimicrobial therapy might be important in the rehabilitation of wild animals to prevent rise in the prevalence of antimicrobial resistant bacteria.

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1. Introduction

The potential for marine mammals to be a source of disease for humans has been recognized since the 1970s (Smith et al., 1978). Marine mammals are known carriers of zoonotic bacteria including *Brucella* spp., *Vibrio* spp., *Leptospira* spp., *Escherichia coli*, *Mycobacterium* spp., and *Salmonella enterica*, with reports of some of these bacteria having antimicrobial resistance (Smith et al., 1978; Johnson et al., 1998; Cowan et al., 2001). There is a public health concern associated with marine mammals due to their close proximity to locations where humans recreate and collect food sources such as mussels, clams, and oysters, as well as the potential for exposure of humans caring for animals during rehabilitation.

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Treatment of marine mammals in rehabilitation often involves the use of antimicrobial drugs (Gulland et al., 2001). Release of marine mammals after their rehabilitation is contingent upon the guarantee that they will not pose a threat to the wild marine mammal or human population after their release (National Marine Fisheries Service, 1972). Our ability to make this guarantee is currently limited, as our knowledge of the effects of release of rehabilitated individuals on wild populations is not known. There is little information on the effects of drug usage during rehabilitation on the antibiotic resistance of bacterial flora of marine mammals. The hypothesis tested in this study was that antimicrobial usage during rehabilitation of northern elephant seals would increase the prevalence and spectrum of antimicrobial resistance in *E. coli* within their gastrointestinal tract.

2. Materials and methods

2.1. Animals, antimicrobial treatments and sampling

Between February and July in 2003 and 2004, 196 juvenile northern elephant seals were found stranded live along the California coast (35°59′N 121°30′W to 37°42′N 123°05′W) and transported to The Marine Mammal Center (TMMC) in Sausalito, CA, USA for rehabilitation. Seals were considered stranded based on the following criteria: depression, dehydration and/or unwillingness to return to sea (Gerber et al., 1993). When collecting a stranded animal, information gathered by the stranding team included: date, time, and location of stranding, condition at stranding, activity level, and harassment by humans or animals. Seals were kept in isolation pens away from other animals until they were examined by the veterinary staff who determined age, weight, cause(s) of stranding, clinical conditions, and course of treatment (Colegrove et al., 2005). Seals were then moved into pens containing a pool with up to five other seals based on husbandry needs and were therefore housed with seals with varying disease and antimicrobial drug treatment status. All animals were approximately the same age (3–6 months). Seals with clinical signs consistent with infection were placed on an antimicrobial drug based on their symptoms and culture results, no animals were treated solely for the purpose of this study. All data regarding location of the animal, clinical status, laboratory results, and treatment of animals with medication (type, dates, dosage) were recorded in the medical record and dated. Stranded seals were assigned to one of two categories retrospectively: (1) antimicrobial drug treated and released animal and (2) animals not receiving antimicrobial drug treatment that were released. Animals that did not survive or were not sampled at release from TMMC were removed from the study. This study complied with the Marine Mammal Protection Act and Institutional Animal Use and Care protocols of the University of California, Davis.

2.2. Isolation of *E. coli*

Stranded seals that were brought to TMMC had rectal swabs collected during physical restraint for admission and release physical examinations. Rectal swabs were placed in Cary–Blair transport medium (BD Diagnostics, Franklin Lakes, NJ, USA) and held at 4 °C until processing within 48 h of collection. All culture and antimicrobial sensitivity testing was performed at the Microbiology Laboratory, William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis. *Escherichia coli* isolates were identified based on standard procedures (Bopp et al., 2003). Briefly, swabs were inoculated directly on to MacConkey agar (Hardy Diagnostics, Santa Maria, CA, USA) and incubated overnight without carbon dioxide at 37 °C. Up to three isolates which showed characteristic phenotypic appearance of *E. coli* on MacConkey agar, which included fermentation of lactose and precipitation of bile salts, were selected. Isolates were subcultured on to 5% defibrinated sheep blood agar and incubated at 37 °C with carbon dioxide. Identification of isolates as *E. coli* was based on a negative reaction on spot oxidase test, positive for spot test indole production, acid/acid with gas production in triple sugar iron (TSI) agar and urease negative on Christensen's urea agar. Forty-three presumptive *E. coli* isolates were further verified as this species using API 20E strips (Biomérieux, Durham, NC, USA). Based on these findings, the remainder of the isolates were identified solely on the basis of phenotypic appearance on MacConkey agar, spot tests, TSI agar, and urea agar findings.

2.3. Antimicrobial sensitivity testing

When possible, antimicrobial susceptibility testing was performed on three *E. coli* isolates from each fecal sample, using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI), formally known as the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 2002a,b). Four to five well-isolated colonies were used to inoculate 2 ml BHI broth and incubated at 37 °C without carbon dioxide for 2–6 h. The broth culture was added drop-wise to 0.85% NaCl to a McFarland Standard of 0.5 as determined by nephlemeter; 10 μl of this suspension was added to cation-adjusted Mueller Hinton-broth and Sensititre® plates (Sensititre®; Trek Diagnostic Systems, Inc., Westlake, OH, USA) were inoculated with 50 μl of broth in each well. The plates were incubated at 37 °C without carbon dioxide overnight and a minimum inhibitory concentration (MIC) determined for each antimicrobial drug. Antimicrobial drugs on the tray were amikacin (AMK), amoxicillin-clavulanic acid (AMC), ampicillin (AMP), cefazolin (CFZ), ceftiofur (CFT), ceftizoxime (ZOX), chloramphenicol (CHL), enrofloxacin (ENR), gentamicin (GEN), tetracycline (TET), ticarcillin-clavulanic acid (TIM), and trimethoprim-sulfamethoxazole (SXT). Methods used for performing and interpreting antimicrobial sensitivity tests were based on CLSI standards using the criteria for animal isolates (National Committee for Clinical Laboratory Standards, 2002a) for all antimicrobial drugs except for ceftizoxime and trimethoprim-sulfamethoxazole in which the human criteria were used (National Committee for Clinical Laboratory Standards, 2002b); no standards have been established for elephant seals. Interpretations for sensitive (*S*), intermediate resistance (*I*) and resistance (*R*) for drugs tested were as

follows (in µg/ml): AMK, $S \leq 16$, $I = 32$, $R \geq 64$; AMC, $S \leq 8$, $I = 16$, $R \geq 32$; AMP, $S \leq 8$, $I = 16$, $R \geq 32$; CFZ, $S \leq 8$, $I = 16$, $R \geq 32$; CFT, $S \leq 2$, $I = 4$, $R \geq 8$; ZOX, $S \leq 8$, $I = 16–32$, $R \geq 64$; CHL, $S \leq 8$, $I = 16$, $R \geq 32$; ENR, $S \leq 0.5$, $I = 1–2$, $R \geq 4$; GEN, $S \leq 4$, $I = 8$, $R \geq 16$; TET, $S \leq 4$, $I = 8$, $R \geq 16$; TIM, $S \leq 16$, $I = 32–64$, $R \geq 128$; SXT, $S \leq 2$, $I = 4$, $R \geq 8$. E. coli strains ATCC 25922 and 35218 were used for quality control weekly. A seal was considered to have resistant E. coli if one or more isolates were either intermediate or fully resistant to one or more of the 12 antimicrobial drugs.

2.4. Statistical analysis

Significant differences amongst the prevalence of antimicrobial resistance of fecal E. coli isolated from admitted and released seals were determined using Fisher's exact test (StatXact 4.0.1, Cytel Software Corp., Cambridge, MA, USA). For the data presented in Table 1, the maximum number of antimicrobial drugs that an isolate of E. coli was resistant to for each seal was modelled. Hence, the isolate from each seal that had resistance to the maximum number of antimicrobial drugs was chosen for the analysis. For all statistical analyses, a P-value of 0.05 or less was considered to be significant. MIC_{50} and MIC_{90} were calculated separately for E. coli isolated at admission and release. Cluster analysis was then used to convert the antimicrobial resistance data into a categorical variable suitable for ordinal logistic regression (Berge et al., 2005). Rather than using the MIC values from 12 different antimicrobials as 12 separate outcome variables, cluster analysis was used to identify groups or clusters of E. coli isolates that exhibit relatively similar patterns of MIC values for the panel of antimicrobials, resulting in a single categorical outcome variable. In addition, this method allowed trends to be reported using the actual MIC values for each antimicrobial drug. The MCLUST extension to S-PLUS 2000 statistical software (Insightful Corp., Seattle, WA, USA) was used to develop these clusters or groups of collinear resistance patterns, based upon the MIC values of the twelve antimicrobial agents (Farley and Raftery, 2000, 2002). In each cluster the median, mean, and standard deviation were reported for each antimicrobial drug. Using these clusters that denoted increasing levels of antimicrobial resistance as the outcome variable, ordinal logistic regression (Stata 7, College Station, TX, USA) was used to

identify factors linked with rehabilitation that were significantly associated with fecal E. coli being more resistant based on CLSI standards. Ordinal logistic regression, as with many statistical regression models assumes that all observations are independent. Our data structure may have violated this assumption given that one to three isolates of E. coli were analyzed from each seal at both admittance and release, potentially resulting in isolates from the same seal being correlated with respect to antimicrobial resistance (i.e., post-antimicrobial drug therapy). As a consequence, a modified version of the ordinal logistic regression model was used, which allowed for standard errors associated with each risk factor to be adjusted for this potential lack of independence for within-seal isolates of fecal E. coli.

Finally, given that each isolate of E. coli was either susceptible or resistant to one or more of the antimicrobial agents based on CLSI standards, negative binomial regression (Stata 7) was used to identify factors linked with rehabilitation that were significantly associated with fecal E. coli being resistant to increasing numbers of different antimicrobials ($n = 0–12$). Early analyses indicated that Poisson regression did not properly model the variance around the mean, requiring the use of negative binomial regression. The data structure may have violated the assumption that all observations were independent given that one to three isolates of E. coli were analyzed from each seal at both admittance and release. As a consequence, a modified version of the negative binomial regression model was used which allowed for standard errors associated with each risk factor to be adjusted for this potential lack of independence for within-seal isolates of fecal E. coli.

3. Results

3.1. Seal information and treatment

In this 2-year study, 99 seals were sampled at admission and release from rehabilitation. Animals were kept in isolation and not treated with antimicrobial drugs until they were first sampled. Treatment of animals commonly included trimethoprim-sulfadiazine for respiratory and skin infections, enrofloxacin for animals with signs of septicemia and/or disseminated intravascular coagulation, and amoxicillin for animals with leptospirosis

Table 1
Prevalence and extent of antimicrobial resistance of E. coli isolated from northern elephant seals being admitted and released (treated with antimicrobials vs. seals not being treated) from rehabilitation

Maximum number[a] of antimicrobial resistant to:	Prevalence in seals (%)				P-Value[b]	
	Admit ($n = 99$)	Release			Admit vs. all release	Treated vs. no treatment
		All ($n = 99$)	Treated ($n = 54$)	No treatment ($n = 45$)		
None	61 (61.6)	24 (24.2)	7 (13.0)	15 (33.3)	–	–
Any resistance	38 (38.4)	77 (77.8)	47 (87.0)	30 (66.7)	<**0.001**	**0.027**
1	27 (27.3)	3 (3.0)	2 (3.7)	1 (2.2)	<**0.001**	1.000
2	4 (4.0)	15 (15.2)	8 (14.8)	7 (15.6)	**0.014**	1.000
3	2 (2.0)	12 (12.1)	8 (14.8)	4 (8.9)	**0.010**	0.540
≥ 4	5 (5.1)	47 (47.5)	29 (53.7)	18 (40.0)	<**0.001**	0.230

[a] Determined by the most resistant E. coli isolate of the three from each seal.
[b] Significant P-values are bolded.

or neutrophilia with a left shift. Of the 99 seals that were in rehabilitation, 45 animals did not receive antimicrobial treatment and 54 received treatment with drugs such as amoxicillin ($n = 35$), trimethoprim-sulfadiazine ($n = 5$), enrofloxacin ($n = 2$), amoxicillin-clavulanic acid ($n = 1$), and doxycycline ($n = 1$). The remainder of the animals ($n = 10$) were treated with a combination of amoxicillin and one or more other antimicrobial drugs.

3.2. Prevalence of seals with at least one isolate of resistant *E. coli*

When investigating the prevalence of antimicrobial resistant *E. coli* from 99 seals that stranded and were rehabilitated at TMMC, seals were more likely to have *E. coli* that were either sensitive to the antimicrobials tested or were resistant to only one antimicrobial at admission compared to seals that were being released from rehabilitation (Table 1). In contrast, the majority of seals being released from TMMC had *E. coli* with multi-drug resistance (Table 1). Seals that were not treated with antimicrobial drugs while in rehabilitation were just as likely to harbor antimicrobial resistant *E. coli* to the same number of drugs as seals that did receive antimicrobial drug treatment (Table 1).

3.3. *E. coli* prevalence and antimicrobial resistance patterns

The most common antimicrobial drugs that *E. coli* isolated from seals at admission to TMMC were resistant to, were ampicillin and tetracycline (Table 2). There was a significantly higher prevalence of resistance to nearly all antimicrobials tested against in fecal *E. coli* isolated at the time of release compared to at admission, with some of the shifts being reflected when comparing MIC_{50} and MIC_{90} values (Table 2). When comparing prevalence of resistance in treated to that in non-treated seals, the only drugs that showed significance were ampicillin, tetracycline, and trimethoprim-sulfamethoxazole (Table 2).

3.4. Admit and release *E. coli* antimicrobial resistance clustering and risk analysis

Based on the Bayesian information criteria (Farley and Raftery, 2000, 2002), four clusters of antimicrobial resistance among our population of 566 admittance and release *E. coli* isolates were identified, with increasing levels of resistance going from Cluster 1 to Cluster 4 (Table 3). Clusters 1 and 2 did not have any MIC values that were in the range of being resistant and the difference between these two clusters was a higher mean chloramphenicol MIC value in Cluster 2. *Escherichia coli* in Cluster 3 were intermediate to fully resistant to ampicillin and tetracycline. Cluster 4 contains *E. coli* with increasing MIC values and resistance to seven antimicrobial drugs. Clusters 1 and 2 consisted mostly of *E. coli* from admit swab samples, whereas Clusters 3 and 4 consisted of *E. coli* from release swabs (Table 3).

The cutoff points for the ordinal logistic regression model presented in Table 4 were -0.221, 0.736, and 2.879 for $k = 1, 2$, and 3, respectively. These cutoff points (k) enter the calculation for the probability of an isolate of *E. coli* to belong to either Cluster 1 through Cluster 4 as: $\text{Pr}(\text{Cluster } i) = \text{Pr}(k_{i-1} < \beta x + u \leq k_i)$, where $k_0 = -\infty$, $k_1 = -0.221$, $k_2 = 0.736$, $k_3 = 2.879$, $k_4 = +\infty$, βx are the risk factor coefficients, and u is a logistically distributed random error term. For example, given that a seal had been at the TMMC for 30 days, during which time it was treated with trimethoprim-sulfadiazine, then the probability that an *E. coli* in the fecal material from such a seal would exhibit a Cluster 1 resistance pattern would be the probability that $(0.021 \times 30 + 1.13 + u) \leq -0.221$, or equivalently, $u \leq -1.981$. Given that u is logistically distributed, the probability is calculated as $1/(1+e^{1.981}) = 0.12$.

The primary risk factor that was associated with the probability that a seal had *E. coli* in Clusters 3 or 4 (strains with multidrug resistance patterns) compared to clusters harboring less resistant *E. coli* (Clusters 1 and 2) was time at the facility (Figs. 1 and 2 and Table 4). This increase in

Table 2

MIC_{50}, MIC_{90}, and prevalence (%) of fecal resistant *E. coli* from northern elephant seals being admitted and released (treated with antimicrobials vs. seals not being treated) from rehabilitation to selected antimicrobials

Antimicrobial drug[a]	MIC_{50}[b]		MIC_{90}[b]		Prevalence (%)				P-Value[c]	
	Admit	Release	Admit	Release	Admit ($n = 289$)	Release ($n = 277$)			Admit vs. all release	Treated vs. no treatment
						All ($n = 277$)	Treated ($n = 156$)	No treatment ($n = 121$)		
AMK	2.00	2.00	2.00	2.00	0 (0)	0 (0)	0 (0)	0 (0)	–	–
AMC	4.00	8.00	8.00	**16.00**	9 (3.1)	44 (15.9)	22 (14.1)	22 (18.2)	**<0.001**	0.410
AMP	4.00	**32.00**	16.00	32.00	30 (10.4)	184 (66.4)	117 (75.0)	67 (55.4)	**<0.001**	**<0.001**
CFZ	2.00	2.00	2.00	8.00	8 (2.8)	7 (2.5)	3 (1.9)	4 (3.3)	1.000	0.700
CFT	0.25	0.25	0.50	0.25	4 (1.4)	1 (0.4)	1 (0.6)	0 (0)	0.370	1.000
ZOX	0.50	0.50	0.50	0.50	3 (1.0)	1 (0.4)	1 (0.6)	0 (0)	0.620	1.000
CHL	8.00	4.00	8.00	**32.00**	10 (3.5)	37 (13.4)	23 (14.7)	14 (11.6)	**<0.001**	0.480
ENR	0.25	0.25	0.25	**1.00**	10 (3.5)	27 (9.7)	19 (12.2)	8 (6.6)	**0.003**	0.150
GEN	0.50	0.50	1.00	2.00	6 (2.1)	24 (8.7)	16 (10.3)	8 (6.6)	**<0.001**	0.390
TET	2.00	2.00	**32.00**	**32.00**	46 (15.9)	101 (36.5)	67 (42.9)	34 (28.1)	**<0.001**	**0.010**
TIM	8.00	8.00	8.00	**64.00**	9 (3.1)	61 (22.0)	38 (24.4)	23 (19.0)	**<0.001**	0.310
SXT	0.25	**8.00**	0.25	**8.00**	17 (5.9)	145 (52.3)	95 (60.9)	50 (41.3)	**<0.001**	**0.002**

[a] Drug abbreviations—AMK: amikacin; AMC: amoxicillin-clavulanic acid; AMP: ampicillin; CFZ: cefazolin; CFT: ceftiofur; ZOX: ceftizoxime; CHL: chloramphenicol; ENR: enrofloxacin; GEN: gentamicin; TET: tetracycline; TIM: ticarcillin-clavulanic acid; SXT: trimethoprim-sulfamethoxazole.

[b] Mean MIC values which are intermediate resistant or resisant, based on CLSI guidelines, are bolded.

[c] Significant P-values are bolded.

Table 3
Median, mean[a] and standard deviation (S.D.) MIC values for cluster groups of E. coli isolated from seals at admit and release from rehabilitation

Cluster	n	% from admit seals	Antimicrobial drug[b] median and mean MIC (S.D.)											
			AMK	AMC	AMP	CFZ	CFT	ZOX	CHL	ENR	GEN	TET	TIM	SXT
1	181	67.4	2.00 1.67 (0.68)	4.00 3.30 (1.02)	2.00 2.60 (0.97)	2.00 2.00 (0.00)	0.25 0.22 (0.08)	0.50 0.50 (0.00)	4.00 3.90 (0.44)	0.25 0.25 (0.00)	0.50 0.55 (0.19)	1.00 1.36 (0.51)	8.00 8.00 (0.00)	0.25 0.25 (0.00)
2	115	76.5	2.00 1.52 (0.51)	4.00 3.88 (1.04)	4.00 3.17 (1.02)	2.00 2.00 (0.00)	0.25 0.26 (0.11)	0.50 0.50 (0.00)	8.00 8.00 (0.00)	0.25 0.25 (0.00)	0.50 0.50 (0.19)	2.00 1.75 (0.44)	8.00 8.00 (0.00)	0.25 0.25 (0.00)
3	199	32.2	2.05 1.88 (1.64)	8.00 7.54 (3.95)	**32.00** **24.11** (12.49)	2.00 2.93 (1.71)	0.25 0.28 (0.11)	0.50 0.50 (0.04)	8.00 9.71 (9.62)	0.25 0.29 (0.12)	0.50 0.70 (0.89)	2.00 **15.27** (15.25)	8.00 13.31 (7.55)	5.00 4.15 (3.86)
4	71	21.1	2.00 1.82 (0.71)	**16.00** **13.66** (7.38)	**32.00** **30.20** (6.75)	8.00 10.82 (8.69)	0.25 0.94 (2.55)	0.50 2.43 (8.71)	8.00 11.94 (10.66)	0.50 **7.62** (7.87)	0.50 **11.77** (14.75)	**32.00** **25.99** (12.22)	**64.00** **46.08** (30.46)	**8.00** **7.13** (2.47)

[a] MIC values which are intermediate resistant or resisant, based on CLSI guidelines, are bolded.
[b] Drug abbreviations—AMK: amikacin; AMC: amoxicillin-clavulanic acid; AMP: ampicillin; CFZ: cefazolin; CFT: ceftiofur; ZOX: ceftizoxime; CHL: chloramphenicol; ENR: enrofloxacin; GEN: gentamicin; TET: tetracycline; TIM: ticarcillin-clavulanic acid; SXT: trimethoprim-sulfamethoxazole.

Table 4
Ordinal logistic regression model for the effect of rehabilitation of northern elephant seals on the risk of harboring fecal E. coli that are in a cluster with increasing levels of antimicrobial resistance[a]

Variable	OLR Coefficient	P-value[b]	95% CI[b]
Duration at TMMC[c]:			
Days	0.021	<0.001	(0.013, 0.028)
Treatment			
Trimethoprim-sulfadiazine			
No[d]	–		
Yes	1.13	<0.001	(0.65, 1.60)

[a] Ordinal logistic regression model for the probability of an isolate of E. coli to exhibit either Cluster 1 through Cluster 4 resistance pattern as function of risk factors (e.g., antimicrobial treatment) and the cutoff point values.
[b] Adjusted for potential lack of independence between isolates of E. coli cultured from the same seal.
[c] Effect of spending time at TMMC was modeled in units of day.
[d] Referent condition for the ordinal logistic regression model.

the prevalence of multidrug resistant E. coli also occurred in seals that had no antimicrobial treatment. Treatment with trimethoprim-sulfadiazine was associated with an approximate doubling of the probability that a seal had E. coli in Cluster 4 compared to E. coli from less resistant clusters ($p < 0.001$) (Table 4 and Fig. 1).

With respect to a quantitative interpretation of the negative binomial regression model shown in Table 5, exponentiating the risk factor coefficients gives the predicted mean number of antimicrobial agents an E. coli would be resistant to. For example, given that a seal had been at the TMMC for 30 days, during which time it was treated with amoxicillin, then we would predict E. coli from such seals would on average exhibit resistance to ($e^{-0.42+0.046\times30-0.0003\times900+0.36}$) $\approx$3 antimicrobial agents. The number of days at the TMMC rehabilitation facility ($p < 0.001$) and a recent history of treatment with amoxicillin ($p = 0.05$) or enrofloxacin ($p = 0.03$) while at
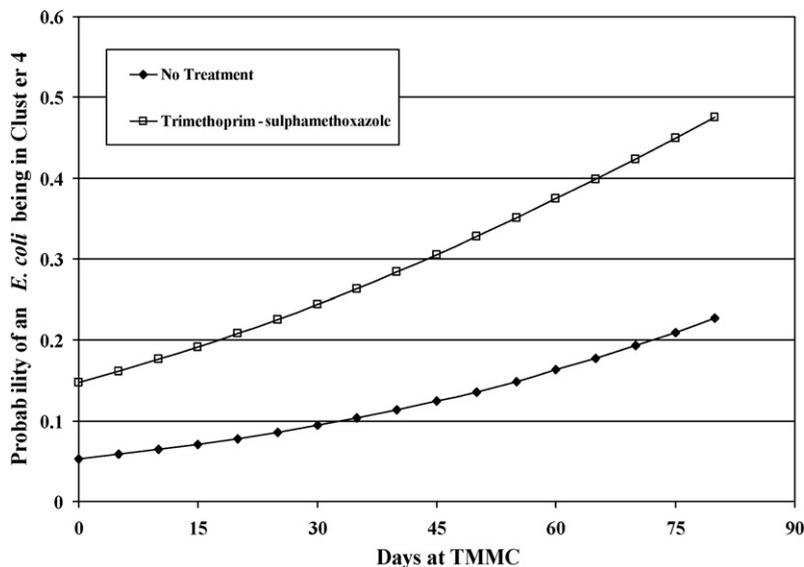
Fig. 1. Probability that an E. coli from a seal will be in Cluster 4 depending on antimicrobial treatment with trimethoprim-sulfadiazine and days at TMMC.
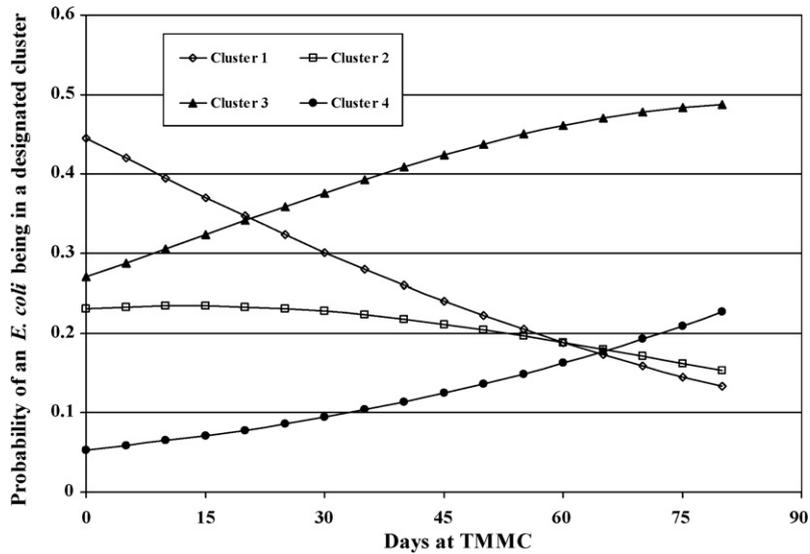
Fig. 2. Probability that an *E. coli* from a seal that did not receive antimicrobial treatment will be in one of the Cluster groups depending on days at TMMC.

the facility were both associated with fecal *E. coli* being intermediate or fully resistant to larger numbers of antimicrobials (Table 5 and Fig. 3).

4. Discussion

This study found that stranding, exposure to a rehabilitation facility, and the use of antimicrobial drugs to treat clinical conditions present at the time of stranding were all associated with increases in antimicrobial resistance of *E. coli* in northern elephant seals. Half of the seals in this study required antimicrobial drugs to treat various clinical conditions. The use of broad-spectrum antimicrobials can select for and promote resistance in environmental and commensal

bacteria which can then cause nosocomial infections (McEwen and Fedorka-Cray, 2002; Hosein et al., 2002; Trott et al., 2004).

In addition to observing antimicrobial resistance in seals that were treated with antimicrobials during rehabilitation, resistance also rapidly developed in seals that were not treated. This suggests that the general environment of the TMMC rehabilitation center was contaminated with resistant *E. coli* that were transmitted between seals during the rehabilitation process. For each additional day that a seal was at TMMC, the probability that its fecal *E. coli* exhibited the higher and multidrug resistance profile of Cluster 3 or 4 increased by 0.1–0.4% (Fig. 2). This spread of resistant bacteria to seals that were not treated while being rehabilitated at the TMMC is
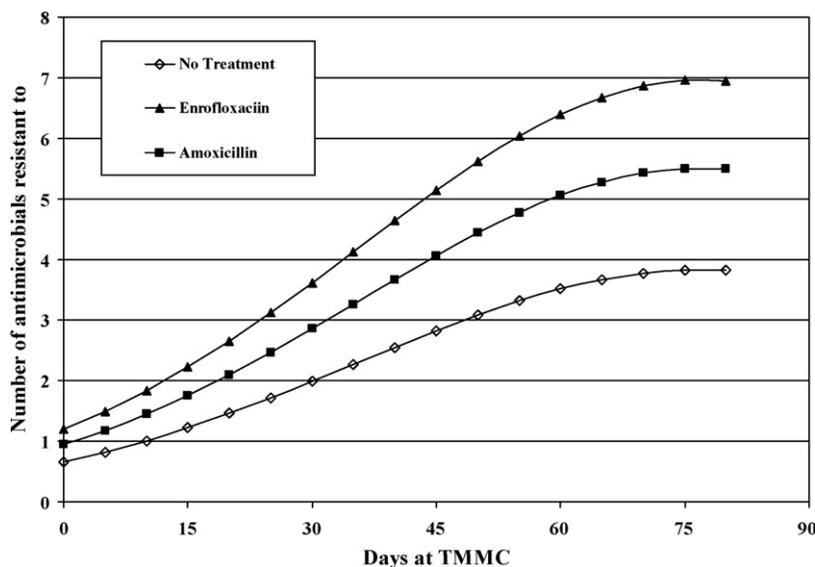

Fig. 3. The effect of antimicrobial treatment and days at TMMC on the number of antimicrobials to which an *E. coli* isolate from a seal is resistant.

Table 5
Negative binomial regression model for the effect of rehabilitation of northern elephant seals on the risk of fecal E. coli having increasing antimicrobial resistance to an increased number of antimicrobials[a]

Risk factor	Coefficient	P-value[b]	95% CI[b]
Duration at TMMC:			
Days	0.046	<0.001	(0.034, 0.057)
Days^2	−0.0003	<0.001	(−0.0004, −0.0002)
Treatment:			
Amoxicillin			
No[d]	–		
Yes	0.36	0.05	(0.005, 0.72)
Enrofloxacin			
No[d]	–		
Yes	0.60	0.03	(0.045, 1.15)
Constant for the model	−0.42	0.06	(−0.86, 0.022)

[a] Resistance to an antimicrobial agent determined by an minimum inhibitory concentration (MIC) that equaled or exceeded the intermediate resistant status as determined by CLSI. Each isolate of E. coli could be resistant to up to 12 antimicrobials.
[b] Adjusted for potential lack of independence for isolates of E. coli cultured from the same seal.
[c] Effect of time at TMMC was modeled as days and days squared to allow, if significant, a polynomial association between days and increasing levels of antimicrobial resistance.
[d] Referent condition for the negative binomial regression model.

similar to the situation observed in human and veterinary hospitals which often have patients with nosocomial infections with antimicrobial resistant bacteria (Sanchez et al., 2002; Hosein et al., 2002). In a study looking at the effect of antimicrobial treatment on coliform resistance in pigs, even the bacteria in control pigs eventually acquired or were replaced by bacteria with increased resistance, which was attributed to cross-contamination through minimal contact with treated pigs (Wiuff et al., 2003). Due to the high case-load, with up to 100 seals housed between March and June, TMMC might provide an optimal environment for spread of resistant bacteria among seals in the same pen or adjacent pen or through movement of animals. The spread of resistant bacteria could also occur via staff that clean the pens and feed the animals, fomites, or through the water supply and drainage system.

Besides being exposed to antimicrobial resistant E. coli, seals and bacteria may also be exposed to antimicrobials present in the environment. The penam-penicillins, cephalosporins, beta-lactamase inhibitors, sulfonamides, and aminoglycosides are eliminated by the kidneys therefore high levels of the agent, often in the active form, can be found in the urine (Prescott et al., 2000). Tetracyclines and fluoroquinolones are not only found in the urine, but also can be excreted in the bile and therefore feces (Prescott et al., 2000). Exposure to low levels of antimicrobials can increase resistance by selecting for more resistant bacterial species and killing resident flora (Dzidic and Bedekovic, 2003). In addition to exposure to antimicrobials, seals could be exposed to biocides, such as chlorhexidine, used at TMMC for cleaning and sterilization purposes. Antimicrobial resistant bacteria may be less susceptible to biocides, and in turn, biocides may promote antimicrobial resistance in bacteria (Russell, 2002).

With a majority of seals leaving TMMC with multi-drug resistant bacteria, the potential impact that these animals may have on the environment must be considered. In humans, it is thought that a short course of an antimicrobial, like ampicillin, can cause resistant bacteria to persist in the feces for 3 months (Cunha, 2000) and pigs treated with enrofloxacin excreted antimicrobial resistant coliforms for at least 2 weeks (Wiuff et al., 2003). Seals from TMMC are released at Point Reyes National Seashore within several hundred feet of other marine mammals and may join them within minutes of release. Despite this, E. coli isolated from seals at Point Reyes were completely sensitive to all antimicrobial drugs tested (unpublished data). Approximately 133 seals were released in 2003 and 2004 into a population of over 80,000 in California (National Marine Fisheries Service, 2000). The environmental load of resistant E. coli that is contributed by the feces of these released seals is likely small compared to the entire E. coli population present in the marine ecosystems due to run-off from human septage, wildlife, domestic animals, farms and surface water.

In conclusion, juvenile northern elephant seals that stranded and went through the rehabilitation process were found to harbor E. coli with high levels of antimicrobial resistance upon release back into the wild. Despite evidence of low levels of antimicrobial resistance in free-ranging (Stoddard et al., 2008) and stranded seals, there is still a concern that rehabilitated seals may disseminate antimicrobial resistant commensal and pathogenic bacteria once released into back into the wild. The results of this study provide evidence that increased levels of hygiene and appropriate use of antimicrobial therapy might be important in the rehabilitation of wild animals. Further studies would be appropriate to determine the modes and methods of preventing the dissemination of antimicrobial resistance during rehabilitation, mechanisms of persistence of resistant bacteria in the population, and the risk factors that expose stranded seals to antimicrobial resistant bacteria.

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