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AEROBIC ORAL AND RECTAL BACTERIA OF FREE-RANGING STELLER SEA LION PUPS AND JUVENILES (*EUMETOPIAS JUBATUS*) IN ALASKA

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ABSTRACT: Bacteriologic cultures from oral, rectal, and lesion samples from free-ranging Steller sea lion (SSL, *Eumetopias jubatus*) pups and juveniles in Alaska (2001–2005) were examined to determine frequency of infection by a specific subset of common and pathogenic aerobic bacteria. Associations between isolated bacteria and age, sex, body condition, location, and sampling season were investigated. *Salmonella* spp. isolates were further evaluated to determine spatial clustering ($n=48$) and to identify serovars ($n=13$) and antimicrobial susceptibility patterns ($n=11$). We sampled 356 SSL pups ($n=272$) and juveniles ($n=84$), and identified 988 isolates of 13 bacterial genera of specific interest. *Pasteurella* spp. (43.8%), beta-hemolytic *Streptococcus* spp. (30.6%), and *Mannheimia* spp. (18.2%) were the most commonly isolated oral bacteria ($n=499$ isolates), whereas *Escherichia coli* (47.6%), beta-hemolytic *E. coli* (32.4%), *Salmonella* spp. (10.4%), and *Campylobacter* spp. (7.8%) were the most frequently isolated rectal bacteria ($n=460$ isolates). *Salmonella* was most commonly found in pups from western stocks and in samples collected during fall/winter seasons. A significant *Salmonella* cluster was detected at the Perry Island haulout. Five serovars were isolated: Enteritidis, Infantis, Newport, Reading, and Stanley. Pulsed-field gel electrophoresis provided evidence that *Salmonella* isolates were most likely being maintained within the SSL population in Alaska.

Key words: Alaska, bacteria, *Campylobacter* spp., *Escherichia coli*, *Salmonella* spp., Steller sea lion.

INTRODUCTION

The Steller sea lion (SSL; *Eumetopias jubatus*) population is divided into genetically distinct eastern and western stocks, corresponding to a geographic boundary at 144°W longitude (60°N, 144°W; Bickham et al., 1996). The western stock has declined by over 80% since the late 1960s, whereas the eastern stock has increased slightly in recent years (Raum-Suryan et al., 2002; Pitcher et al. 2007). Because of the decline, the western SSL stock was listed as endangered under the Endangered Species Act (ESA) in 1997; the eastern stock is listed as threatened (NRC, 2003). Despite research efforts and protection measures, there is no evidence to conclusively identify the causes of the decline. Factors proposed include prey limitation/nutritional stress, predation by killer whales, climate change, toxins, entanglement in marine debris, incidental or intentional take by humans, and infectious diseases (Rea et al., 1998; National Research Council [NRC], 2003; Burek et al., 2005a; Atkinson et al., 2008).

Because bacterial agents commonly cause morbidity and mortality in free-ranging and captive pinnipeds, information on common potentially pathogenic bacterial flora in actively managed populations, like the SSL, can aid policy decision making and potentially could improve biological monitoring. Similarly, bacterial pathogens and antimicrobial drug resistance should be monitored in

species that may serve as early warning systems for pathogen pollution.

Our goal was to identify common bacteria of the rectal and oral cavities of free-ranging SSL pups and juveniles from the eastern and western stocks, as well as potentially pathogenic bacteria that could be limiting population recovery. Recovery efforts for SSLs include intensive monitoring of pups and juveniles. These efforts presented an opportunity to characterize a subset of the rectal and oral aerobic flora and obtain baseline data about the strains and antimicrobial susceptibility of *Salmonella* spp. encountered in SSL, 2001–2005.

MATERIALS AND METHODS

Study population

Rectal and oral samples were collected from free-ranging SSL ($n=356$) from 31 rookeries and haulouts in Alaska from 2001 to 2005 (Fig. 1). Most individuals sampled were judged to be healthy by a veterinarian, but if an animal had a lesion, additional samples ($n=40$ from 37 SSLs) were collected from potentially infected sites. Of 37 SSL from which additional samples were taken, postmortem examinations were performed on six pups under the direction of a veterinary pathologist (KAB). Collection sites for samples for bacteriologic analysis included lung, pleural fluids, abscesses, and aqueous humor.

Steller sea lion pups <3 mo old were captured by hand on land with the use of hoop nets; older animals were captured by scuba divers with the use of an underwater noose capture technique (Raum-Suryan et al., 2004). Age was estimated based on body size, weight, dental measurements, and time of year when captured according to the estimated pupping date of early June (Burek et al., 2005a). Animals under 1 yr old were classified as pups and those between 1 and 3 yr old as juveniles. Body condition was scored by conformation and palpation of blubber thickness and bony protuberances (Burek et al., 2005b). Animals were classified as in poor body condition if they were emaciated with scant subcutaneous fat/evidence of muscle wasting and in good body condition if they had moderate to abundant subcutaneous fat. Seasons were fall/winter (September–March) and spring/summer (April–August). The SSL study population was divided into eastern stock (southeast Alaska; $n=126$) and western stock (Prince William Sound through the Aleutian Island chain; $n=230$) according to genetic criteria (Bickham et al., 1996).

Specimen collection and bacteriology

Rectal and oral cavities were sampled using dacron swabs (Fisher Scientific, Pittsburgh, Pennsylvania, USA), which were transferred to tryptic-soy broth with 15% glycerol, frozen on dry ice, and stored at −80 C. Samples were shipped to the Washington Animal Disease Diagnostic Laboratory (WADDL, Pullman, Washington, USA) on dry ice. Specific bacterial organisms were targeted for evaluation based on two criteria: 1) previous identification as pathogens in other marine mammals or domestic animals, and 2) previous identification as potential pathogens of SSL at the WADDL. We did not attempt to identify every isolate comprehensively, and in the vast majority of cases, there were bacteria that we ignored once screening indicated they were not organisms of interest.

Oral samples ($n=334$) were cultured to detect *Actinobacillus* spp., *Bordetella* spp., beta-hemolytic streptococci, coagulase-positive staphylococci, *Erysipelas* spp., *Mannheimia* spp., and *Pasteurella* spp.. Samples were inoculated onto nonselective Columbia agar with 5% sheep blood (Hardy Diagnostics, Santa Maria, California, USA) with a streak of *Staphylococcus aureus* as a nurse colony. Colistin-nalidixic acid supplemented blood agar (CNA plates, Hardy Diagnostics) was also inoculated to select for gram-positive organisms. The plates were incubated at 35 C in a 5% CO_2-enriched atmosphere for ≥5 days. Identification was performed by standard bacteriologic algorithms (Murray et al., 2003). Candidate gram-negative organisms (*Actinobacillus*, *Bordetella*, *Mannheimia*, and *Pasteurella*) were screened by tests for the production of indole from tryptophan, ability to reduce nitrate, production of oxidase, and production of urease. Fermentation of glucose or lactose/sucrose and production of hydrogen sulfide was detected using triple-sugar-iron slants. Further biochemical characterization was done with API 20E and API 20 NE strips (Biomerieux, Hazelwood, Missouri, USA). Candidate gram-positive organisms (beta-hemolytic streptococci, coagulase-positive staphylococci, *Erysipelas*) were isolated and screened by patterns of hemolysis, production of catalase, and gram staining characteristics. Further biochemical characterization of staphylococci was done by determining production of coagulase and API Staph strips (Biomerieux). Further biochemical

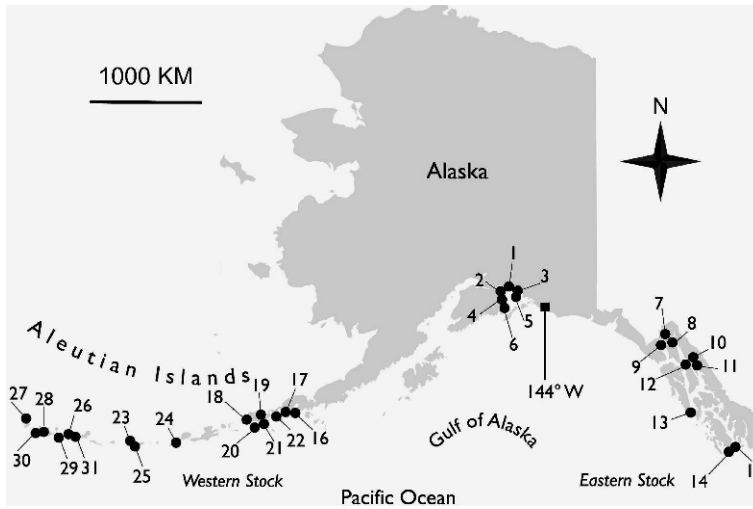

FIGURE 1. Distribution of Steller sea lion (*Eumetopias jubatus*) rookeries and haulouts ($n=31$) where animals tested positive ($n=10$) and negative ($n=21$) for *Salmonella* spp. on rectal swabs collected from Steller sea lions from the southern coast of Alaska, 2001–2005. Locations positive for *Salmonella* spp. include Glacier Island (1), Perry Island (2), Benjamin Island (8), Little Island (9), Southwest Brothers (11), Clubbing Rock (16), Akun Island (18), Ugamak Island (19), Cape Morgan (21), Adak Island (29). Locations negative to *Salmonella* spp. include Seal Rocks (3), Procession Rocks (4), The Needle (5), Point Elington (6), Gran Point (7), Sunset Island (10), West Brothers (12), Hazy Island (13), North Rocks (14), Cape Horn Rocks (15), South Clubbing Rock (17), Tigalda Island (20), Aiktak (22), Amlia Island (23), Yunaska Island (24), Seguam Island (25), Silak Island (26), Semisopochnoi (27), Kanaga Island/Ship Rock (28), Kagalaska Island (30), Little Tanaga Island (31). The western and eastern SSL stocks are separated at 144°W longitude.

characterization of beta-hemolytic streptococci was done with Lancefield grouping and API 20 Strep strips (Biomerieux). *Erysipelas* was identified by production of hydrogen sulfide and API Coryne strips (Biomerieux).

We cultured 341 rectal samples to detect *Campylobacter* spp., *Edwardsiella* spp., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Yersinia* spp. For isolation of *Campylobacter*, samples were inoculated onto *Brucella* agar with 5% sheep blood, vancomycin, amphotericin B, and cefoperazone (Campy-CVA agar, Hardy Diagnostics) and incubated at 42°C for a ≥7 days in a microaerobic atmosphere containing 84% N_2, 10% CO_2, and 6% O_2. Isolates were stained with Victoria Blue and evaluated microscopically. Isolates were tested for sensitivity to nalidixic acid and cephalothin (30 μg disks each) and characterized biochemically with the API Campy strips (Biomerieux). The other organisms all belong to the *Enterobacteriaceae* and were screened by growth on MacConkey's agar, ability to ferment lactose, production of indole from tryptophan, and production of oxidase. Fermentation of glucose or lactose/sucrose and production of hydrogen sulfide were detected with the use of triple-sugar-iron slants. Where appropriate, identification to genus and species was performed with API 20E strips (Biomerieux).

Rectal samples were also enriched for *Salmonella* by inoculation into tetrathionate broth with iodine. After 24 hr incubation, the tetrathionate broth was subcultured to MacConkey's and Brilliant Green agars (Hardy Diagnostics), and candidate isolates screened biochemically. *Salmonella* isolates were serogrouped with slide agglutination tests with the use of commercial polyvalent and serogroup-specific *Salmonella* A-I and Vi antisera (Difco Laboratories, Detroit, Michigan, USA). Serovar determination ($n=13$) was performed on representative isolates from each serogroup by the National Veterinary Services Laboratory in Ames, Iowa, USA. Selected *Salmonella* isolates ($n=11$) were tested for antimicrobial susceptibility by microbroth dilution methods (National Committee for Clinical Laboratory Standards [NCCLS], 2002). Antimicrobials used for susceptibility testing included amikacin, ampicillin, amoxicillin/clavulanic acid, apramycin, ceftiofur, cefazolin, cefoxitin, cephalothin, chloramphenicol, chlortetracycline, enrofloxacin, florfenicol, gentamicin, imipenem, marbofloxacin, orbifloxacin, oxytetracycline, penicillin, spectinomycin,

sulfachloropyridazine, sulfadimethoxime, sulfathiazole, tetracycline, ticarcillin, ticarcillin/clavulanic acid, and trimethoprim/sulfamethoxazole.

Escherichia coli isolates were subcultured on blood agar to distinguish hemolytic from nonhemolytic strains. *Escherichia coli* virulence genes were detected by multiplex polymerase chain reaction (PCR) for the F5 fimbriae, heat-stable enterotoxin (*sta*), intimin (*eae*), shiga toxins (*stx1*, *stx2*), and the shiga toxin associated with porcine edema disease (*stx2e*) according to standard methods (Franck et al., 1998).

Pulsed-field gel electrophoresis

Restriction enzyme profiles of *Salmonella enterica* isolates were compared with the use of a standard pulsed-field gel electrophoresis (PFGE; Ribot et al., 2006), with minor modifications. Genomic DNA from 35 *Salmonella* isolates was digested with the restriction enzyme *Xba*I (30 U, Invitrogen, Carslbad, California, USA) for 4 hr at 37 C. The PFGE was performed on a CHEF-DRIII PFGE apparatus (BioRad, Hercules, California, USA) with the use of the following parameters: Separation on a 1% agarose (Seakem Gold agarose) gel in 0.5X tris-borate-EDTA at 14°C at 6 V/cm with pulse times ramped from 2.2 to 63.8 sec for 19 hr. Gels were stained with ethidium bromide and photographed with UV transillumination with the use of a gel documentation system (Syngene Gene Genius Bioimaging System, Synoptics, Cambridge, UK). Digital images were analyzed with the use of Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram illustrating relative similarity of PFGE profiles was generated in Bionumerics (Applied Maths) with the use of the unweighted-pair group method with arithmetic mean algorithm for cluster analysis of Dice similarity coefficients with a position tolerance of 2%.

Statistical analysis

Chi-square analysis was used to evaluate associations between bacterial culture results and SSL age, sex, stock, body condition, and season of sampling using SPSS® statistical software (SPSS, Inc., Version 15, ©2006, Chicago, Illinois, USA). Stratified analyses were considered where appropriate, following univariate analyses. For variables with small sample sizes, Fisher's exact test was used to measure the significance of associations with the use of Epi Info® statistical software (Epi Info, Ver. 6, 1993, Center for Disease Control and Prevention, Atlanta, Georgia, USA). Odds ratios (OR) and 95% confidence intervals (CI) were used to estimate the strength of association between each rectal and oral bacterial isolate, and age, sex, stock, body condition and season of sampling.

Locations (rookeries and haulouts) were mapped with the use of ArcView GIS® software for all SSL swabs, as well as locations from which *Salmonella* spp. were isolated. Spatial clustering of rectal swabs positive for *Salmonella* spp. was evaluated with the use of SaTScan® statistical software (Kulldorff, 1997). The Poisson model was chosen to identify *Salmonella* clusters in relation to the population at risk that were negative for *Salmonella* during the study period using age as a covariate. Scanning was performed for times with high rates (50% population at risk) in 1-yr aggregations within 15-km-radius circles. The estimate of 15-km radius was based on the assumption that Steller sea lion pups and juveniles are more likely to travel short distances between near-shore areas adjacent to haulouts (Raum-Suryan et al., 2004). Statistical significance of clusters was calculated with Monte Carlo hypothesis testing and 999 iterations. Secondary clusters were not allowed to have geographic overlap.

RESULTS

Bacteriology

Bacterial isolates ($n=988$) were obtained from samples collected from the oral cavity, rectum, and other anatomical sites of 356 SSL pups and juveniles. Thirteen bacterial genera were identified, and 13 of 50 *Salmonella* isolates from 10 locations were serotyped (Fig. 1). Similarly, antimicrobial sensitivity was performed on 11 of the 50 *Salmonella* isolates. Isolates were selected for antimicrobial testing based on serogroup representation and sampling location. No statistically significant associations were found between sex and any oral or rectal bacterial isolate. Although age was not significantly associated with any oral isolates, there were age-specific associations with isolates (*E. coli*, beta-hemolytic *E. coli*, *E. coli eaeA*–gene positive, *Salmonella* spp., and *Campylobacter* spp.) from rectal swabs. Therefore, the remaining analyses for stock, body condition, and season were stratified by age.

Pasteurella spp. (219/499, 43.8%), beta-hemolytic *Streptococcus* spp. (153/499, 30.6%), and *Mannheimia* spp. (90/499, 18.0%) were the most common isolated oral bacteria from the 334 SSL. Several species of *Streptococcus* and *Staphylococcus* were identified by API system and biochemical procedures. Isolation techniques were aimed at identifying potential pathogens; therefore, beta-hemolytic streptococci were of specific interest. Although nonhemolytic streptococci were not specifically investigated, 11 isolates were incidentally identified. Based on the Lancefield serogrouping, beta-hemolytic streptococci group F was the predominant type detected (72/111 isolates, 65%), followed by group G (31/111 isolates, 28%), group C (5/111, 4.5%), and group A (3/111, 2.7%). Biochemical evaluation of *Staphylococcus* spp. revealed that 10 isolates were coagulase positive. Other bacteria incidentally isolated from oral samples were *Moraxella* spp., *Vibrio* spp., and *Pseudomonas* spp. Seven *Vibrio* spp. isolates were identified from six pups and one juvenile from four rookeries (Akun Island, Ugamak Island, Cape Morgan, and Clubbing Rocks) in the Aleutian Islands in September 2001. One *Vibrio parahaemolyticus* was identified from an injured pup on Benjamin Island during February of 2004. Seven *Moraxella* and four *Pseudomonas* isolates were recovered from pups. Unusual growth of aerobic bacteria from oral samples included two isolates of *E. coli* and two of *Salmonella*, one group B and the other group C2.

We recovered 460 rectal isolates from 341 SSL pups and juveniles. *Escherichia coli* (219/460 isolates, 47.6%), beta-hemolytic *E. coli* (149/460 isolates, 32.4%), *Salmonella* spp. (48/460, 10.4%), and *Campylobacter* spp. (36/460, 7.8%) were the most frequently isolated bacteria. Screening for *Edwardsiella* spp. and *Yersinia* spp. was consistent, but only three *Edwardsiella* and one *Yersinia* isolates were identified. Other bacterial growth incidentally identified from rectal samples included three isolates of *Pasteurella* spp., one *Corynebacterium* spp., one *Enterococcus* spp., two *Proteus* spp., and seven nonhemolytic streptococci, six of which were members of the *Streptococcus bovis/equinus* group. Evaluation of *E. coli* virulence genes by PCR revealed 45 rectal isolates positive for the *eaeA* (intimin) gene; all were negative for shiga toxin genes (*stx1*, *stx*2, *and stx*2*e*), attachment pilus (F5), and heat-stable enterotoxin (*sta*).

Salmonella groups B (18/48 isolates, 37.5%) and C2 (17/48, 35.4%) were the predominant serogroups, followed by serogroup D (12/48, 25%) and serogroup C1 (1/48, 2%). Thirty-one *Salmonella* isolates were recovered from SSL pups and juveniles at rookeries and haulouts in Prince William Sound. Ten *Salmonella* isolates were recovered from rookeries and haulouts along the Aleutian Islands, and seven were recovered in southeast Alaska. Thirteen *Salmonella* isolates of five serovars were identified (Table 1). *Salmonella* isolates ($n=11$) were susceptible to ampicillin, ceftiofur, and enrofloxacin, and all isolates tested for resistance to imipenem, tetracycline, chloramfenicol, florfenicol, cephalothin, and gentamicin ($n=9$) were susceptible to those antimicrobials. One of nine tested for resistance to trimethoprim–sulfamethoxazole was resistant to this antimicrobial. Specific resistance patterns for each *Salmonella* isolate tested are shown in Table 1.

When adjusted for age, two significant clusters were detected between 2001 and 2005. The highest risk cluster was at Perry Island haulout (Fig. 1), which contained 29 observed *Salmonella* cases when 4.5 were expected ($P<0.001$). Of the 29 cases, three animals were positive to three isolates: *Salmonella* Enteritidis, *Salmonella* Newport, and *Salmonella* Reading. At Perry Island, SSL pups had a higher risk of being positive for *Salmonella* spp. when compared with SSL juveniles (RR=14.3). A second cluster was identified at the Cape Morgan rookery where six *Salmonella* cases were observed when 1.6 were expected ($P=0.17$).

TABLE 1. Distribution of *Salmonella* serovars and antimicrobial resistance of rectal swabs collected from Steller sea lion (*Eumetopias jubatus*) pups and juveniles from the southern coast of Alaska, by year, age group, and location.

Animal by year	Region[a]	Location (Fig. 1)	Salmonella serovars	Antimicrobial resistances
2001				
Pup 1	AI	Ugamak Island (19)	Stanley	
Pup 2	AI	Cape Morgan (21)		Sulfadimethoxime
Pup 3	PWS	Glacier Island (1)	Newport	Sulfadimethoxime, spectinomycin
2002				
Pup 4	SE	Benjamin Island (8)	Infantis	Spectinomycin
2003				
Pup 5	SE	Cape Horn Rocks (15)[b]	Reading	Sulfadimethoxime, spectinomycin
Pup 6	PWS	Perry Island (2)	Enteritidis	Sulfadimethoxime, spectinomycin, penicillin, sulfathiazole, tiamulin, tilmicosin
Pup 7	SE	Benjamin Island (8)	Reading	Sulfadimethoxime, spectinomycin, penicillin, sulfathiazole, tiamulin, tilmicosin
Pup 8		Perry Island (2)	Enteritidis	Gentamicin, spectinomycin, sulfadimethoxime, trimethoprim-sulfamethoxazole, penicillin
Juvenile 1	SE	Little Island (9)	Reading	Sulfadimethoxime, spectinomycin, penicillin, sulfathiazole, tiamulin, tilmicosin
2005				
Pup 9	PWS	Perry Island (2)	Reading	Sulfadimethoxime
Pup 10	PWS	Perry Island (2)	Newport	Sulfadimethoxime
Pup 11	PWS	Perry Island (2)	Enteritidis	Sulfadimethoxime
Pup 12	AI	Lake Point Adak (29)	Stanley	
Juvenile 2	PWS	Glacier Island (1)	Newport	

[a] AI = Aleutian Islands; PWS = Prince William Sound; SE = southeast Alaska.

[b] Sample collected from oral cavity.

Aerobic culture results for the 40 samples collected from clinically suspect lesions are presented in Table 2. Of the 6 *Streptococcus* skin isolates, two were nonhemolytic and four were beta-hemolytic (one group F, one group A, and one group C). Complete postmortem examinations on the six pups showed that two 2-4-wk-old pups had severe pleuropneumonia and sepsis as the primary cause of death, with isolations of beta *E. coli* from lung and pleural fluid in one and from pleural fluid and aqueous humor in the other. Beta-hemolytic streptococci were also isolated from the lung and aqueous humor; however, the beta-hemolytic *E. coli* was considered to be the primary cause of the sepsis and pleuropneumonia of these two pups. Another pup had an abscess in the pectoral region and bronchopneumonia from which *Salmonella* Enteritidis was isolated. Another had a bite wound and osteomyelitis with mixed culture suggestive of a bite wound, another died of acute trauma, and the other of aspiration of milk related to capture and with no significant bacterial cultures.

Pulsed-field gel electrophoresis

Salmonella serotype–specific chromosomal PFGE fingerprinting patterns were produced for 35 isolates from 31 pups and three juveniles incorporating all five *Salmonella* serogroups identified (Fig. 2). *Salmonella* serogroup C2 from Perry and Glacier

TABLE 2. Number of free-ranging Steller sea lion (*Eumetopias jubatus*) pups and juveniles ($n=37$) from the southern coast of Alaska from which bacteria were isolated from swabs of potentially infected sites. Cultures were attempted to detect bacteria on 40 samples collected from 37 sea lions.

Bacteria	Conjunctivitis ($n=9$)	Skin lesions[a] ($n=20$)	Fluids[b] ($n=6$)	Pneumonia ($n=2$)
Corynebacterium spp.	2	5		
Mannheimia spp.	1			
Pasteurella spp.	2	2		
Streptococcus spp.	3	6	2	1
Staphylococcus spp.		1		
Beta *Escherichia coli*		1	3	1
Salmonella Enteritidis		1		1

[a] Included ulcers, punctures, lacerations, abscesses, and suspected fungal infections.

[b] Included abdominal, synovia, aqueous humor, vaginal discharges, tracheal mucus, and pleural fluid.

Island haulouts had similar fingerprinting patterns (SSL 1–12) and a slightly different profile was observed in an isolate from SW Brothers haulout (SSL 13). Serogroup C2 isolates were identified as *Salmonella* Newport. Most of these isolates were recovered from rectal swabs collected from one juvenile and 12 pups during 2001, 2002, and 2005. One *Salmonella* serogroup C1 was identified as *Salmonella* Infantis, which had been collected from the rectal cavity of a pup from Benjamin I. haulout in 2002 (SSL 14).

We identified two serotypes of group B: *Salmonella* Stanley and *Salmonella* Reading. Two fingerprinting patterns of *Salmonella* Stanley were identified, one from the rectal cavity of a pup from Adak Island rookery in 2005 and the other collected from a pup from the Ugamak Island rookery in 2001 (SSL 15 and 16). *Salmonella* Reading had three fingerprinting patterns, one from both oral and rectal swabs collected from two pups and one juvenile from SW Brothers and Little Islands haulouts and Cape Horn Rocks rookery during 2002 and 2003 (SSL 17–19), the second one from the rectal cavity of a juvenile from SW Brothers in 2002 (SSL 20), and the other from the rectal cavity of two pups from Perry Island haulout in 2005 (SSL 21–22). All 12 *Salmonella* serogroup D isolates from Benjamin and Perry Islands haulouts had the same fingerprinting pattern. All of these isolates from rectal swabs collected from SSL pups during 2003 and 2005 were identified as *Salmonella* Enteritidis (SSL 23–34).

DISCUSSION

Marine mammals are susceptible to a wide range of bacterial pathogens, but little is known about the normal microbiologic flora of many species or the pathologic significance of specific bacteria in marine mammal populations (Higgins, 2000). Infectious agents have been postulated as a factor in the reduction of reproductive success and increase in morbidity and mortality of Steller sea lions in Alaska (NRC, 2003; Burek et al., 2005a). However, specific study of bacterial prevalence has been hampered by the vastness and remoteness of SSL habitat, making consistent sampling of live animals and recovery of dead animals difficult.

Although the majority of the bacteria reported in this study exist in the natural environment and may be part of the normal transient flora of SSL, some can also act as opportunistic agents causing disease in naive populations or animals with compromised immune or nutritional status (Higgins, 2000; Dunn et al., 2001). *Pasteurella*, *Mannheimia*, and beta-hemolytic *Streptococcus* spp. were commonly isolated from oral swabs from SSL pups and juveniles, findings that were expected, because these potential pathogens are frequent commensal organisms of the upper respiratory tract of wild

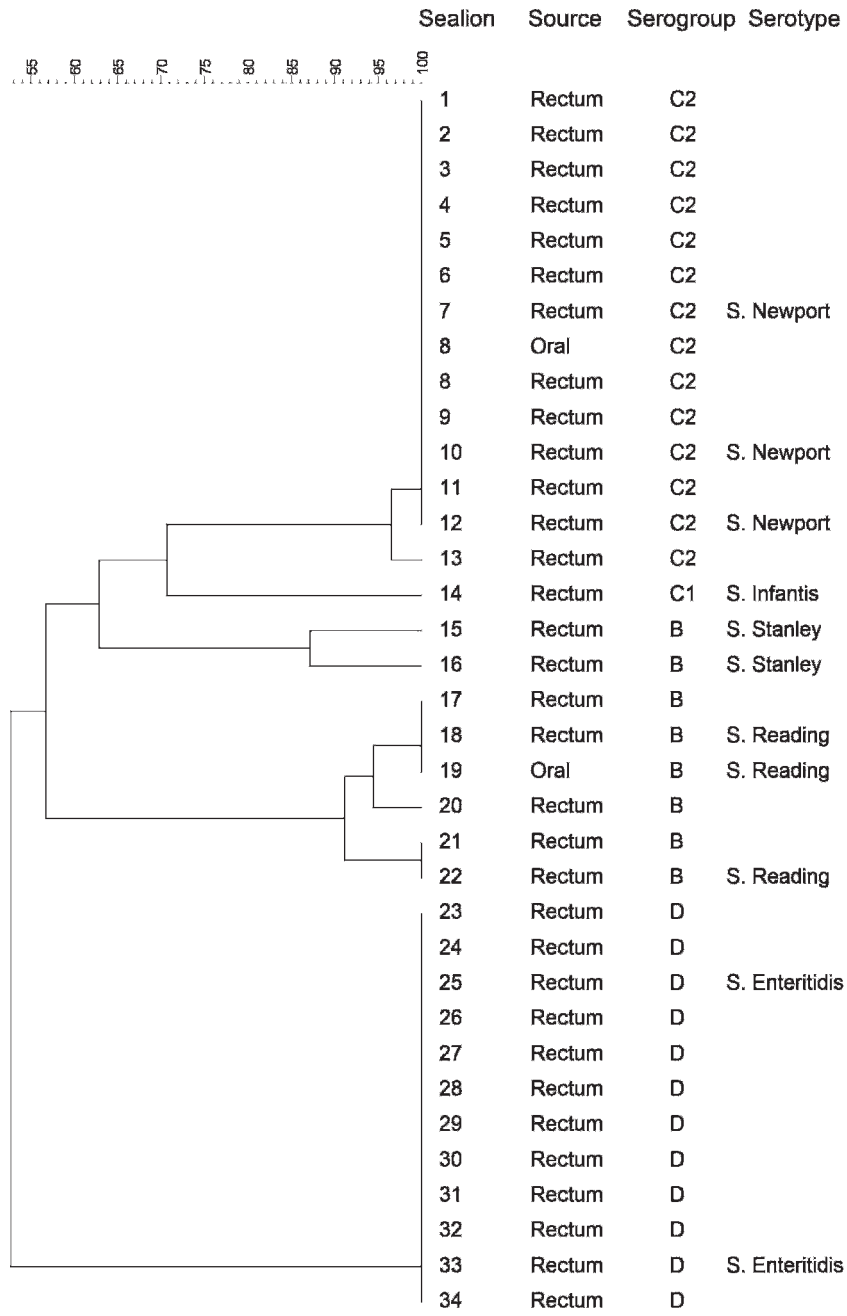

FIGURE 2. Dendrogram based on pulsed field gel electrophoresis of *Salmonella* spp. genomic DNA digested with restriction endonuclease *Xba*I from rectal and oral swabs collected from Steller sea lion (*Eumetopias jubatus*) pups and juveniles from the southern coast of Alaska, 2001–2005 ($n=34$).

and domestic animals (Thornton et al., 1998; Higgins, 2000; Rudolph et al., 2003).

Multiple streptococcal organisms have been isolated from sick and healthy free-ranging sea lions and seals (Thornton et al., 1998; Higgins, 2000; Johnson et al., 2006; Goldstein et al., 2007), but the beta-hemolytic streptococci appear to be more

frequently associated with disease, such as pneumonia and septicemia (Baker et al., 1989; Thornton et al., 1998; Higgins, 2000; Johnson et al., 2006). Beta-hemolytic streptococci may be opportunistic pathogens of SSL pups considering our findings that pups in poor body condition were nearly three times more likely to be positive for beta-hemolytic *Streptococcus* spp. when compared to pups in good body condition (OR=2.9; 95% CI=1.26–6.88).

We isolated *Vibrio* spp. from oral samples from two areas in 2001 and 2004. Several species of *Vibrio* have been recovered from aquatic invertebrates, fish, and marine mammals in estuarine and marine environments (Dunn et al., 2001; Buck et al., 2006; Miller et al., 2006). *Vibrio parahaemolyticus* can cause acute gastroenteritis in humans after ingestion of contaminated seafood (McLaughlin et al., 2005) as well as wound infections in areas of broken skin (Daniels et al., 2000). Increased water temperature was an important factor associated with an outbreak of *V. parahaemolyticus* on a cruise ship sailing from Prince William Sound during July 2004 (McLaughlin et al., 2005), just after our identification of *V. parahaemolyticus* in an injured pup from Benjamin Island. As global temperatures increase, this pathogen should be monitored carefully for increases in prevalence and potential effects on human health and wildlife populations.

Escherichia coli were isolated from the majority of the rectal samples collected from SSL pups and juveniles (63.5% and 66.7%, respectively). Beta-hemolytic *E. coli* was the second most commonly isolated bacterium in SSL pups and juveniles (49.4% and 24.4%, respectively), but pups were three times more likely to be positive for beta-hemolytic *E. coli* when compared to juveniles (OR=3.0; 95% CI=1.66–5.6). Similar results were described by Goldstein et al. (2007), who found *E. coli and* beta-hemolytic *E. coli* in rectal samples collected from SSL from Resurrection Bay and Prince William Sound, Alaska. They may also be opportunistic pathogens; Thornton et al. (1998) and Johnson et al. (1998) frequently isolated this organism from abscesses, wounds, ocular and urethral discharges, and the umbilicus of live and stranded pinnipeds and seals from Californian waters. Our evaluation of potentially pathogenic *E. coli* strains indicated that SSL pups and juveniles are not likely to carry common virulence factors, such as F5 fimbriae and *sta* toxin that are associated with enteric disease in domestic animals (DebRoy and Maddox, 2001). However, juveniles were more likely to harbor the *eaeA* (intimin) gene than were pups (OR=2.1; 95% CI=1.01–4.28), and this gene occurred more commonly in the eastern stock (OR=6.8; 95% CI=1.71–29.09). Studies performed with cattle have shown that some *E. coli eae* strains can cause attaching and effacing (AE) lesions in the enterocytes, inducing diarrhea in calves (Wieler et al., 1996; Holland et al., 1999). We did not identify other virulence factors, such as shiga toxins, which can produce enterohemorrhagic *E. coli* disease and elicit AE lesions. Further investigations may be warranted to evaluate the genotypic and phenotypic characteristics, as well as the epidemiology, of *E. coli* strains in the southeast Alaska ecosystem where the *eae* gene was more prevalent.

Campylobacter jejuni, *Campylobacter coli*, and *Campylobacter lari* have been reported in marine mammals (Stoddard, et al. 2005; Stoddard et al., 2007), and the genus is a common enteric pathogen of birds, domestic animals, and humans. Recent studies have identified a new, potentially marine mammal–specific, species in northern elephant seals (*Mirounga angustirostris*) and SSL named *Campylobacter insulaenigrae* (Foster et al., 2004; Goldstein et al., 2007; Stoddard et al., 2007). Similar to intimin-positive *E. coli*, *Campylobacter* was more commonly found in SSL pups in the eastern stock (OR=2.7; 95% CI=1.03–7.09), and juveniles were twice as likely to be positive (OR=2.4; 95% CI=1.16–4.94). This higher prevalence in

the eastern stock could be associated with the increase in the SSL population in southeast Alaska (Pitcher et al. 2007). Because *Campylobacter* is transmitted through the fecal–oral route, crowding at rookeries and haulouts may facilitate transmission and infection. Wild birds could be another source of infection, because gulls serve as a reservoir (Kapperud and Rosef, 1983; Quessy and Messier, 1992).

In Alaska, *Salmonella* was associated with mortality in northern fur seals (*Callorhinus ursinus*) from the Pribilof Islands (Jellison and Milner, 1958), and has been sporadically detected in dead SSL from south-central Alaska (Spraker and Bradley, 1996). Similar to our findings, *Salmonella* Reading and *Salmonella* Newport were recently isolated from rectal swabs collected from healthy juveniles of the western stock (Goldstein et al., 2007). However, we found a higher prevalence of *Salmonella* as well as an increased diversity of serovars in free-ranging SSL and we are the first to report *Salmonella* Stanley and *Salmonella* Infantis in sea lions.

Pups from the western stock were nearly six times more likely to have *Salmonella* isolated from rectal swabs than pups from the eastern stock (OR=5.8; 95% CI=1.99–23.18). Several studies have proposed that the western stocks of SSL may be nutritionally stressed because of a reduction in prey abundance or in quality of prey in the Gulf of Alaska and Aleutian Islands (Trites and Donelly, 2003). Although current literature supports the theory that nutritional stress could affect the fitness and health of the western stock of SSL (Trites and Donelly, 2003), our analysis and ongoing research by the Alaska Department of Fish and Game did not find a difference in body condition between SSL in the eastern and western stocks (L. D. Rea, Alaska Department of Fish and Game, pers. com.). The major cluster of *Salmonella* cases was detected at Perry Island near the eastern edge of the western stock, rather than in the Aleutian chain. This location also had the most diverse serovars of *Salmonella*. Sources of infection contributing to this cluster at Perry Island likely included fecal contamination from wild bird reservoirs (Gilmartin et al., 1979), supported by the fact that seabirds and marine mammals have identical fingerprinting patterns within *Salmonella* serovars when they coexist (Palmgren et al. 2000, Stoddard et al., 2008). Another possibility is that SSL maintain *Salmonella* as nonpathologic flora. Supporting this theory is the finding of similar PFGE restriction patterns within *Salmonella* groups B (Stanley and Reading), C2 (Newport), and D (Enteritidis), suggesting that the same clones were present and maintained among SSL across years. Although these findings may indicate that SSL pups and juveniles have *Salmonella* as endemic flora, testing of adult SSL would be useful in assessing shedding and transmission from adults to offspring.

There was an association between *Salmonella* isolation and sampling season (Fisher exact test, $P<0.001$), with the highest number of positive pups during fall and winter when most SSL are in haulout areas. Because sampled pups were approximately 3–7 mo old and highly dependent on lactating females, it is possible that they acquired *Salmonella* from their shedding mothers. Winter may also be a critical period of restricted energy intake due to dispersed fish distribution and increased energy demands for mothers nursing late lactation pups (Trites and Porter, 2002). This time of the year may predispose nutritionally stressed mothers to shed *Salmonella* and facilitate exposure of naive animals on haulouts.

Salmonella spp. are also important zoonotic pathogens that cause disease in mammals, birds, and reptiles (Smith et al., 2002). Because they can potentially be transmitted through water sources, the identification of *Salmonella* in marine mammal feces, seabirds, and marine invertebrates has raised public health concerns

(Stoddard et al., 2005; Miller et al., 2006). *Salmonella* has been linked to fecal contamination from terrestrial sources not only from densely populated areas, such as the California coast (Stoddard et al., 2005; Miller et al., 2006), but also from remote areas, such as Antarctica (Palmgren et al., 2000). The *Salmonella* groups, B, C1, C2, and D and serovars Infantis, Reading, Newport, and Enteritidis, detected in this study, were also common in feces of humans and gulls, and sled dogs in Alaska (Williams and Dodson, 1960; Cantor et al., 1997). Although we detected similar *Salmonella* groups and serovars between SSL and other terrestrial species, it is unlikely that SSL infections were from terrestrial sources, as positive samples were often from less-populated and more remote and pristine Alaskan ecosystems. However, the possibility that SSL acquired *Salmonella* from wastewater discharges from large cruise ships and small vessels cannot be ruled out (Alaska Department of Environmental Conservation [ADEC], 2002).

Changes of antimicrobial susceptibility and new multidrug-resistant patterns of *Salmonella* spp. in humans and animals have also raised public health concerns (Wright et al., 2005; Foley and Lynne, 2008). In pinnipeds, common enteric pathogens have displayed resistance before or after animals are admitted to rehabilitation centers (Johnson et al., 1998; Smith et al., 2002). Studies with California sea lions and northern elephant seals found that *Salmonella* spp. were resistant or had moderate susceptibility to ampicillin (Johnson et al., 1998; Smith et al., 2002; Stoddard et al., 2005). However, in our study, all our *Salmonella* isolates were susceptible to ampicilli, perhaps suggesting that, in contrast to SSL, live-stranded California sea lions and northern elephant seals may have been exposed to resistant *Salmonella* strains from terrestrial pollution. Similar to our SSL findings, when California sea lions were tested on remote islands off the California coast, antibiotic resistance was not observed for *Salmonella* spp. (Stoddard et al., 2008).

This study has begun to elucidate risk factors associated with the high prevalence and regional differences of *Salmonella* spp. shedding in SSL in remote and more populated near-shore environments. The use and continued development of molecular diagnostic techniques will advance our ability to distinguish strains of *Salmonella* spp. isolated from wildlife and humans sharing ecosystems. Because *Salmonella* has zoonotic potential, this study provides cautionary information for biologists, veterinarians, and technicians handling and collecting samples from SSL. The presence of *Salmonella* and other aerobic bacteria we report complements previous work on the health of juvenile SSLs, and provides baseline data on potential pathogens within the normal oral flora of SSL. This information may be useful in disease outbreak investigations and surveillance programs aimed at recovery of the endangered SSL population.

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