



Aerobic bacterial flora of the vagina and prepuce of California sea lions (*Zalophus californianus*) and investigation of associations with urogenital carcinoma

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Abstract

To investigate the association between genital bacterial infection and urogenital carcinoma in California sea lions (*Zalophus californianus*), vaginal and preputial swabs for bacterial isolation were taken from 148 free-ranging and 51 stranded California sea lions including 16 animals with urogenital carcinoma. Cytological examination of vaginal or preputial smears showed a majority (65.5%, 57/87) of animals examined had mild or no inflammation. Aerobic bacteria were isolated from 116 (78.4%) wild sea lions and 100% of stranded animals. A total of 403 isolates were identified representing 51 unique bacterial species. The median number of isolates per animal increased with age in the wild group, but there was no difference in the number of isolates per animal between wild and stranded adults. The most common bacteria isolated from the wild sea lions were *Psychrobacter phenylpyruvicus* (39 isolates), non-hemolytic *Streptococcus* (35 isolates), *Corynebacterium* spp. (30 isolates), and *Escherichia coli* (20 isolates). More bacterial species were isolated from stranded animals than wild animals (33 versus 26) and there was significantly less growth of *P. phenylpyruvicus*, *Corynebacterium* spp., and *Moraxella*-like spp. in the stranded animals. Beta-hemolytic *Streptococcus* was the only bacterium significantly associated with urogenital carcinomas in California sea lions, but only in females.

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1. Introduction

A high prevalence of urogenital carcinomas has been observed in California sea lions (*Zalophus californianus*) (Gulland et al., 1996). Although the etiology of these carcinomas is unknown, a herpesvirus infection has been detected in all cases examined (Lipscomb et al., 2000; King et al., 2002, Lowenstine, unpublished data). As the etiology of many carcinomas is multifactorial, urogenital bacterial infections and associated inflammation may play a role in the development of these aggressive metastatic carcinomas in California sea lions. In humans, epidemiologic studies of cervical carcinomas suggest that human papillomavirus (HPV) in conjunction with *Chlamydia trachomatis* (Tamim et al., 2002) and other vaginal bacteria are primary agents in the development of these tumors (Guijon et al., 1985). Bacterial infections appear to serve as cofactors in tumor development by reducing local immunity and inducing inflammation thus rendering vaginal epithelial cells more susceptible to other agents (Guijon et al., 1992). To determine the significance of bacterial infections in urogenital carcinogenesis in California sea lions, the normal microbial flora of the urogenital tract must be identified, taking into account variations in the host and environment. However, little is known about the normal urogenital flora of California sea lions or the presence of infectious and sexually transmitted diseases in the species. Johnston and Fung (1971) cultured the vulva and prepuce of only seven wild caught California sea lions isolating *Escherichia coli*, *Aerobacter* spp., *Staphylococcus aureus*, and *Streptococcus fecalis*, all of which are considered potential pathogens. Reproductive disorders such as abortion and neonatal death have been associated with *Leptospira pomona* infection on San Miguel Island, CA (Smith et al., 1974). *Brucella* antibodies are present in marine mammals of the Pacific Ocean (Nielsen et al., 2001) and the organism has been isolated from an adult female California sea lion in rehabilitation (Gulland, personal communication); however, its significance remains unclear. Studies of Steller sea lions (*Eumetopias jubatus*), a species closely related to the California sea lion with overlapping habitat, revealed 49% of animals tested had serologic evidence of *Chlamydia* exposure (Calkins and Goodwin, 1988) and *Chlamydia* (*Chlamydia*) *psittaci* was cultured from an aborted fetus (Bradley et al., 1994). Unfortunately, no

studies have adequately characterized the normal urogenital bacterial flora of different age classes California sea lions.

Without knowledge of the endemic microbial flora of free-ranging animals, understanding the role of bacteria in marine mammal strandings, reproductive disorders, and morbidity and mortality is difficult. In an attempt to understand the pathogenesis of urogenital carcinomas in California sea lions, this study aimed to characterize vaginal and preputial aerobic bacterial flora in different age classes of wild and stranded California sea lions and investigate associations among vaginal and preputial bacterial flora, inflammation, and urogenital carcinoma in this species.

2. Materials and methods

2.1. Study populations

Vaginal and preputial swab specimens were obtained from two groups of California sea lions from January 2001 to March 2003. The first group consisted of apparently healthy free-ranging adult females (>4 years), juveniles (1–3 years) and pups (<1 year) captured during the spring and fall of both years from rookeries on San Miguel Island, CA, and adult males (>4 years) inhabiting Puget Sound, WA in May 2001 and June 2002. The second (stranded) group consisted of debilitated adult California sea lions that stranded along the central and northern California coast due to acute diseases such as trauma and neurotoxin (domoic acid) intoxication and were taken to a rehabilitation center (The Marine Mammal Center, Sausalito, CA). Vaginal and preputial swabs for bacterial culture were obtained from the live or fresh dead (<12 h since death) stranded animals that had not been administered antibiotics prior to sampling. The presence of urogenital carcinoma was diagnosed post mortem in stranded animals by gross and histological examination of tissues.

2.2. Specimen collection

Swabs for bacterial culture were obtained by either the use of Accu-culshure™ guarded collection device (Accu-Med, Pleasantville, NY), a double-guarded swab (Jorgensen Laboratories, Loveland, CO) with

Port-a-CulTM transport tube (BD Biosciences, Cockeysville, MD), or with a vaginal speculum and bacterial swab in Amies transport media (Copan Diagnostics, Corona, CA). The skin around the vulva and prepuce were cleaned with a germicidal solution (NolvasanTM, Fort Dodge Animal Health, Fort Dodge, IA) before insertion of the swab or speculum and each swab was vigorously manipulated to collect mucus and cells. Inoculated swabs in transport media were held at ambient temperature for 1–7 days depending on geographic location of sampling and transported to the microbiology laboratory at the Veterinary Medical Teaching Hospital, University of California (Davis, CA) for aerobic bacterial culture.

Additional sterile applicator swabs were inserted into the vulva or prepuce to obtain epithelial cells and exudates. Material retrieved from the first swab was streaked onto four glass slides and air dried for cytological examination. The remaining swabs were placed in cryovials and frozen at -80°C .

2.3. Bacterial culture procedures

Swabs for bacterial culture were vortexed in 1 ml of brain heart infusion broth. A 0.2 ml aliquot of the broth was plated on several culture media for the isolation of a broad spectrum of aerobic organisms. Tryptic soy agar (TSA) with 5% sheep blood and chocolate agar (for non-selective bacterial isolation), pleuro-pneumonia-like organisms (PPLO) agar with thallium acetate penicillin (for *Mycoplasma* spp.), MacConkey (for enteric bacteria), and Food and Drug Administration (FDA) Campy agars (for *Campylobacter* spp.) were streaked so that a semi-quantitative measure of bacterial growth could be obtained (Hirsh and Wiger, 1977). An aliquot was also inoculated into selenite broth and alkaline peptone water for 24 h before being sub-cultured onto XLT4 and TCBS agars (Hardy Diagnostics, Santa Maria, CA), respectively, for isolation of *Salmonella* spp. and *Vibrio* spp. The TSA, chocolate, and PPLO media were incubated in a 10% CO₂ with air environment. The MacConkey, XLT4, and TCBS plates were incubated under standard atmospheric conditions and the FDA under microaerophilic conditions (Pack-Campylo, AnaeroPack SystemTM, Mitsubishi, Inc., New York, NY). Plates were incubated at 37 °C and examined for growth daily for 5 days. All bacterial isolates were identified and classified into family,

genus, or species according to standardized morphologic and biochemical methods (Jang et al., 1986; Murray, 1999; Hirsh et al., 2004).

2.4. Beta-hemolytic *Streptococcus* characterization

To further investigate associations discovered between beta-hemolytic streptococci and urogenital carcinomas (see Section 3), further characterization of the beta-hemolytic *Streptococcus* isolates was performed using biochemical and serological testing and 16S rDNA analysis. Biochemical testing was accomplished with the API 20 Strep identification system (bioMerieux, Hazelwood, MO) and serologic Lancefield grouping into A, B, C, D, F and G was performed by latex bead antibody agglutination using a Streptex kit (Remel Inc., Lenexa, KS). A representative sample of beta-hemolytic *Streptococcus* isolates was chosen for sequence analysis of 16S rDNA using a single colony from each isolate for DNA extraction. Templates for PCR amplification were extracted from a crude lysis of bacterial colonies, using a QIAamp DNAeasy minikit (QIAGEN, Valencia, CA). Eubacterial primers designated 8FPL and 1492RPL were used to amplify a 292 bp fragment (Foley et al., 1998). PCR products were separated and visualized on an ethidium bromide-stained 1% agarose gel and were then purified using a Microcon[®] centrifuge filter device (Millipore Co, Bedford, MA), as previously described (Johnson et al., 2003). Amplification products were sequenced by Davis Sequencing (Davis, CA) and compared with the database in the National Center for Biotechnology Information (NCBI). To further differentiate species within Lancefield groups, bacitracin susceptibility testing was performed on isolates using bacitracin discs (BBLTM discs, BD Diagnostic Systems) on Muller–Hinton agar containing 5% sheep blood (Swenshon et al., 1998). Any zone of inhibition was interpreted to indicate susceptibility.

2.5. Molecular assays

Molecular assays were used to identify organisms of interest that are often difficult to culture. Vaginal and preputial swabs were tested for the presence of *Chlamydia* and *Chlamydiophila* spp. and *Brucella* spp. using a PCR assay. Bacterial DNA was extracted

from swabs with the QIAamp DNA Mini Kit™ (Qiagen, Valencia, CA) following the manufacturer's instructions. *Chlamydia/Chlamydiophila* spp. were detected using a single pair of primers designed to detect all known members of the family *Chlamydiaceae*. The primers amplified a segment band of 1094 bp using previously described methods (Sykes et al., 1997). *Brucella* spp. were detected using primers and methods designed to amplify the entire *bp26* gene resulting in a 1900 bp product that is unique to marine mammal strains of *Brucella* (Cloeckert et al., 2000). A *Brucella* strain isolated from a stranded California sea lion was used for positive control.

2.6. Cytological examination

Slides for cytological examination were stained with Wright–Giemsa and evaluated for the presence of inflammation, dysplastic cells, and for cytologic features of malignancy. Inflammation was classified as none, mild, moderate, marked, and severe based primarily on the number of cells present and degree of neutrophil karyolysis (degenerative change). Inflammation was classified as purulent, pyogranulomatous or granulomatous based upon the type of inflammatory cells present. Septic inflammation was diagnosed if intracellular bacteria were observed.

2.7. Data analysis

Data collected on each animal sampled included: animal identification number, sex, age, location of capture, date of sampling, degree of cytological inflam-

mation, bacterial culture and PCR results and presence of neoplasia and other diagnosed diseases. Odds ratios with 95% confidence intervals (C.I.), χ^2 , and the median test were calculated using Epi Info 2002 (US Centers for Disease Control and Prevention, Atlanta, GA) and SPSS (SPSS, Inc., Chicago, IL) statistical software. A p -value <0.05 was considered statistically significant.

3. Results

Swabs for bacterial culture were obtained from 148 free-ranging and 35 stranded California sea lions in rehabilitation. An additional 16 animals with urogenital carcinoma, diagnosed at the rehabilitation center, were swabbed for bacterial isolation. The sex and age distribution within the groups of animals is listed in Table 1. Wild caught animals consisted of three age classes, but only adult animals were sampled in the stranded cohort. All carcinoma cases were adult animals. A total of 403 isolates were identified representing 51 unique bacterial organisms. The median number of isolates per animal was 2 (range, 0–7). No growth occurred in the 32 samples from the wild group (24 pups, 3 juveniles, 5 adults) but bacterial growth was present in all samples obtained from the stranded animals.

3.1. Bacterial associations with age

Only data from wild caught animals were used to compare bacterial growth among age classes. Two hundred and fifty-four isolates representing 26

Table 1
Characteristics of California sea lions sampled for vaginal and preputial bacteriology

| Group | Number | Sex | | Bacterial Isolation | Total number isolates | Median isolates per animal | Range | Cytology smears examined | Inflammation | | | | |
|-----------|--------|-----|----|---------------------|-----------------------|----------------------------|-------|--------------------------|--------------|------|----------|--------|--------|
| | | M | F | | | | | | None | Mild | Moderate | Marked | Severe |
| Wild | | | | | | | | | | | | | |
| Pup | 60 | 23 | 37 | 36 | 55 | 1 | 0–3 | 24 | 18 | 4 | – | 2 | – |
| Juvenile | 23 | 6 | 17 | 20 | 34 | 1* | 0–4 | 19 | 7 | 7 | 4 | – | 1 |
| Adult | 65 | 40 | 25 | 60 | 165 | 3* | 0–7 | 39 | 19 | 8 | 5 | 6 | 1 |
| Stranded | | | | | | | | | | | | | |
| Adult | 35 | 11 | 24 | 35 | 106 | 3 | 1–7 | 33 | 16 | 4 | 5 | 6 | 2 |
| Carcinoma | | | | | | | | | | | | | |
| Adult | 16 | 6 | 10 | 16 | 43 | 3 | 1–5 | 8 | 1 | 5 | 2 | – | – |

* Significant difference (median test, $p = 0.001$).

different bacteria species were obtained from the wild California sea lions. Most of the isolates were from adult animals (165/254 isolates, 65.0%) followed by pups (55/254 isolates, 21.7%) then juveniles (34/254 isolates, 13.4%). The median number of isolates per animal was significantly different amongst the age classes and increased with age (Table 1, $p = 0.001$).

The distribution of bacterial growth among the age classes is presented in Table 2. *Psychrobacter phenylpyruvicus* (39 isolates), non-hemolytic *Streptococcus* (35 isolates), and *Corynebacterium* spp. (30 isolates) were the most frequently isolated bacteria from the wild California sea lions and were present in all age classes. *P. phenylpyruvicus* was the predominant bacterium in adults (26 isolates, 38.5%) followed by non-hemolytic *Streptococcus* (23 isolates, 35.5%),

Cornebacterium spp. (20 isolates, 30.8%), non-fermentative gram-negative bacilli (13 isolates, 20.0%) and *Moraxella*-like spp. and *Escherichia coli* (11 isolates each, 16.9%). Similar distributions of bacterial isolates were also evident from pup and juvenile animals. The *Salmonella* isolates from pups and adult females grew in large amounts and all but one of the *Salmonella* isolates were obtained in pure culture.

3.2. Bacterial associations with animal origin

Bacteria isolated from 35 stranded California sea lions are listed in Table 3 with the isolates from wild adults included for comparison. Acute domoic acid toxicity was the cause of stranding in 57.1% (20/35) of the rehabilitation animals followed by acute traumatic

Table 2
Bacterial isolates from the vagina and prepuce of free ranging California sea lions

| Group | Bacteria | Number of isolates | | | |
|--|--|---------------------|--------------------------|-----------------------|--------------------------------------|
| | | Pup ($n = 60$) | Juvenile ($n = 23$) | Adult ($n = 65$) | Total, $n = 148$ (%) ^a |
| Psychrobacter and related organisms | <i>Psychrobacter phenylpyruvicus</i> | 2 | 12 | 25 | 39 (26.4) |
| | <i>Moraxella</i> -like spp. | – | – | 11 | 11 (7.4) |
| | <i>Neisseria</i> -like spp. | – | – | 1 | 1 (0.7) |
| Streptococcus | Non-hemolytic <i>Streptococcus</i> | 9 | 3 | 23 | 35 (23.6) |
| | Beta-hemolytic <i>Streptococcus</i> | – | – | 5 | 5 (3.4) |
| Corynebacterium/Bacillus | <i>Corynebacterium</i> spp. | 5 | 5 | 20 | 30 (20.3) |
| | <i>Bacillus</i> spp. | 2 | – | 3 | 5 (3.4) |
| Non-fermentative gram-negative Bacilli (NFB) | NFB | 6 | 4 | 13 | 23 (15.5) |
| | <i>Flavobacterium</i> -like spp. | – | – | 6 | 6 (4.1) |
| | <i>Pseudomonas stutzeri</i> | 3 | – | – | 3 (2.0) |
| | <i>Stenotrophomonas maltophilia</i> | – | – | 3 | 3 (2.0) |
| Enterobacteriaceae | <i>Escherichia coli</i> | 9 | – | 11 | 20 (13.5) |
| | Haemolytic <i>Escherichia coli</i> | 6 | – | – | 6 (4.1) |
| | <i>Klebsiella pneumoniae</i> | – | – | 5 | 5 (3.4) |
| | <i>Edwardsiella tarda</i> | – | 1 | 4 | 5 (3.4) |
| | <i>Enterobacter cloacae</i> | – | – | 5 | 5 (3.4) |
| | <i>Salmonella</i> spp. | 2 | – | 2 | 4 (2.7) |
| Staphylococcus/micrococcus | Coagulase-negative <i>Staphylococcus</i> | 4 | 4 | 6 | 14 (9.5) |
| | <i>Micrococcus</i> spp. | – | 1 | 1 | 2 (2.0) |
| Other | <i>Mycoplasma</i> spp. | 5 | 3 | 8 | 16 (10.8) |
| | gram-positive rod (catalase-negative) | 1 | 1 | 6 | 8 (5.4) |
| | <i>Actinobacillus</i> -like spp. | – | – | 3 | 3 (2.0) |
| | <i>Shewanella putrefaciens</i> | – | – | 1 | 1 (0.7) |
| | <i>Photobacterium damsela</i> | 1 | – | 1 | 2 (0.7) |
| | <i>Pleisomonas shigelloides</i> | – | – | 1 | 1 (0.7) |
| | <i>Pasteurella</i> -like spp. | – | – | 1 | 1 (0.7) |

^a Percentage of animals with the isolate.

Table 3
Bacterial isolates from the vagina and prepuce of adult free-ranging wild and stranded California sea lions

| Bacteria | Number of isolates | |
|--|--|--|
| | Wild <i>n</i> = 65 (%) ^a | Stranded <i>n</i> = 35 (%) ^a |
| <i>Psychrobacter phenylpyruvicus</i> * | 25 (38.5) | 5 (14.3) |
| Non-hemolytic <i>Streptococcus</i> | 23 (35.4) | 7 (20.0) |
| <i>Corynebacterium</i> spp.* | 20 (30.8) | 1 (2.9) |
| Non-fermentative gram-negative bacilli* | 13 (20.0) | 15 (42.8) |
| <i>Escherichia coli</i> | 11 (17.0) | 11 (31.4) |
| <i>Moraxella</i> -like spp.* | 11 (17.0) | – |
| <i>Mycoplasma</i> spp. | 8 (12.3) | 4 (1.4) |
| Gram-positive rod (catalase-negative) | 6 (9.2) | 8 (22.9) |
| coagulase-negative <i>Staphylococcus</i> | 6 (9.2) | 2 (5.7) |
| <i>Flavobacterium</i> -like spp. | 6 (9.2) | – |
| Beta-hemolytic <i>Streptococcus</i> | 5 (7.7) | 5 (14.3) |
| <i>Klebsiella pneumoniae</i> | 5 (7.7) | 2 (5.7) |
| <i>Enterobacter cloacae</i> | 5 (7.7) | – |
| <i>Edwardsiella tarda</i> | 4 (6.2) | 4 (11.4) |
| <i>Bacillus</i> spp. | 3 (4.6) | 2 (5.7) |
| <i>Stenotrophomonas maltophilia</i> | 3 (4.6) | – |
| <i>Actinobacillus</i> -like spp. | 3 (4.6) | – |
| <i>Salmonella</i> spp. | 2 (3.1) | – |
| <i>Shewanella putrefaciens</i> | 1 (1.5) | 2 (5.7) |
| <i>Neisseria</i> -like spp. | 1 (1.5) | – |
| <i>Micrococcus</i> spp. | 1 (1.5) | – |
| <i>Photobacterium damsela</i> | 1 (1.5) | – |
| <i>Pleisomonas shigelloides</i> | 1 (1.5) | – |
| <i>Pasteurella</i> -like spp. | 1 (1.5) | – |
| <i>Proteus vulgaris</i> | – | 6 (17.1) |
| <i>Aeromonas</i> spp. | – | 4 (11.4) |
| <i>Proteus mirabilis</i> | – | 3 (8.6) |
| <i>Pseudomonas fluorescens</i> | – | 3 (8.6) |
| <i>Pseudomonas putida</i> | – | 3 (8.6) |
| <i>Aeromonas hydrophila</i> | – | 3 (8.6) |
| haemolytic <i>Escherichia coli</i> | – | 2 (5.7) |
| Viridans streptococcus | – | 2 (5.7) |
| <i>Enterobacter</i> -like spp. | – | 2 (5.7) |
| <i>Alcaligenes</i> -like spp. | – | 1 (2.9) |
| <i>Acinetobacter calcoaceticus</i> | – | 1 (2.9) |
| <i>Chryseobacterium</i> -like spp. | – | 1 (2.9) |
| <i>Enterococcus</i> spp. | – | 1 (2.9) |
| <i>Providencia rettgeri</i> | – | 1 (2.9) |
| <i>Morganella morganii</i> | – | 1 (2.9) |
| <i>Citrobacter freundii</i> | – | 1 (2.9) |
| <i>Corynebacterium phocae</i> | – | 1 (2.9) |
| <i>Arcanobacterium phocae</i> | – | 1 (2.9) |
| <i>Pseudomonas aeruginosa</i> | – | 1 (2.9) |

^a Percentage of animals in group with isolate.

* Significantly different between groups (χ^2 , $p < 0.05$).

wounds (8/35, 17.1%), leptospirosis (4/35, 14.3%), and pneumonia and cystitis (3/35, 11.7%). A greater variety of bacterial types (32 versus 24, respectively) were isolated from stranded than free-ranging sea lions. The median number of isolates per animal in the stranded adults was not significantly different from that of the wild adults (Table 1, $p = 0.62$). There was statistically significant less growth of *P. phenylpyruvicus* ($p = 0.012$), *Corynebacterium* spp. ($p = 0.001$) and *Moraxella*-like spp. ($p = 0.01$) in the stranded compared to the wild adults and significantly more growth of non-fermentative gram-negative bacilli ($p = 0.015$) in the stranded group (Table 4).

3.3. Bacterial associations with sex

Bacterial differences between males and females were investigated. There was a statistically significant difference in the median number of isolates between males (median = 3, range 1–7) and females (median = 1, range 0–6) in adult wild animals ($p = 0.028$), but not between male (median = 3, range 2–7) and female (median = 3, range 1–6) stranded adults ($p = 0.67$). No relationship between bacteria species isolated and sex in the stranded animals was observed, but in wild adults, males had statistically significantly more growth of non-hemolytic *Streptococcus* ($p = 0.04$) and *Moraxella*-like species ($p = 0.004$) than females. The *Mycoplasma* spp. isolated from 10.8% (16/148) of the wild animals was most often isolated from males (15/16 isolates were obtained from the prepuce) and the beta-hemolytic streptococci were isolated from males more often than females in both wild and stranded animals (9/10 cases).

3.4. Genital inflammation and urogenital cancer

Vaginal or preputial cytology smears from 82 wild caught animals and 33 stranded animals were of a quality sufficient for evaluation (Table 1). No inflammation was observed in samples from 44 (53.7%) wild animals and 16 (48.5%) stranded animals. Nineteen (23.2%) wild caught animals had mild inflammation, nine (11.0%) moderate, eight (9.8%) marked, and two (2.4%) severe inflammation detected on cytological examination. Fewer stranded animals had mild (12.1%) and moderate (15.2%) inflammation and more had marked (18.8%) and severe

Table 4
Bacteria isolated from California sea lions with urogenital carcinomas and odds of association with the cancers

| Bacteria | Number of isolates | | | |
|--------------------------------------|--------------------------------|------------|-------------------|--------------|
| | <i>n</i> = 16 (%) ^a | Stratified | Odds ^b | (95% C.I.) |
| Beta-hemolytic <i>Streptococcus</i> | 8 (50.0) | Crude | 6.0 | (1.3–29.5) |
| | | Male | 0.9 | (0.1–10.3) |
| | | Female | 34.5 | (2.6–1651.7) |
| Non-hemolytic <i>Streptococcus</i> | 7 (43.8) | Crude | 3.1 | (0.7–13.6) |
| | | Male | 1.3 | (0.8–17.4) |
| | | Female | 5.0 | (0.7–34.8) |
| <i>Escherichia coli</i> | 7 (43.8) | Crude | 1.7 | (0.4–6.7) |
| | | Male | 0.9 | (0.1–10.3) |
| | | Female | 2.4 | (0.4–14.3) |
| Hemolytic <i>Escherichia coli</i> | 3 (18.8) | Crude | 3.8 | (0.4–49.5) |
| | | Male | N/A ^c | |
| | | Female | 2.7 | (0.2–42.6) |
| <i>Arcanobacterium phocae</i> | 2 (12.5) | Crude | 4.9 | (0.2–294.8) |
| <i>Psychrobacter phenylpyruvicus</i> | 2 (12.5) | Crude | 0.9 | (0.1–6.1) |
| <i>Corynebacterium</i> spp. | 2 (12.5) | Crude | 6.2 | (0.3–375.1) |
| <i>Enterococcus</i> spp. | 2 (12.5) | Crude | 4.9 | (0.2–294.8) |
| Gram-positive rod | 2 (12.5) | Crude | 0.5 | (0.04–2.9) |
| <i>Shewanella putrefaciens</i> | 1 (6.3) | Crude | 1.1 | (0.02–22.7) |
| <i>Mycoplasma</i> spp. | 1 (6.3) | Crude | 0.5 | (0.01–5.9) |
| Non-fermentative bacilli | 1 (6.3) | Crude | 0.5 | (0.01–5.9) |
| <i>Erysipelothrix</i> -like spp. | 1 (6.3) | N/A | | |
| <i>Campylobacter</i> -like spp. | 1 (6.3) | N/A | | |
| <i>Lactobacillus</i> spp. | 1 (6.3) | N/A | | |
| <i>Salmonella enteritidis</i> | 1 (6.3) | N/A | | |
| <i>Vibrio alginolyticus</i> | 1 (6.3) | N/A | | |

^a Percent of cases with isolate.

^b Odds ratio with 95% confidence intervals (C.I.) for bacterial association with carcinoma.

^c Not applicable.

(6.1%) inflammation than was observed in the wild animals. No bacterial species were statistically associated with degree of cytological inflammation. However, the two adult wild females with *Salmonella* spp. had marked inflammatory responses. *Mycoplasma* spp. isolated from the wild adult males appears to be a non-pathogenic species since 7 of 8 had none or mild inflammation on cytology. Cytology smears from eight carcinoma cases examined showed mild to moderate epithelial hyperplasia in all cases and moderate to marked inflammation in seven of the cases, but no cytologic evidence of urogenital carcinoma.

3.5. Bacterial associations with urogenital cancer

The culture results from 16 sea lions with urogenital carcinoma are listed in Table 3. Bacterial

growth occurred in all cases resulting in 43 isolates from 17 different bacterial species. A median of three (range, 1–5) isolates per animal were obtained which was not statistically different from animals without cancer.

Beta-hemolytic *Streptococcus* was the most frequently isolated bacterium from carcinoma cases (8 isolates, 50.0%). Female California sea lions with urogenital carcinomas were 34.5 times (95% CI, 2.6–1651.7) more likely to have beta-hemolytic streptococci than females stranded for other causes, but males with carcinomas were not (OR = 0.9, 95% C.I., 0.1–10.3) more likely to have beta-hemolytic streptococci. No other bacteria were significantly associated with the presence of urogenital carcinomas.

The phylogenetic and phenotypic characteristics of beta-hemolytic *Streptococcus* isolates from stranded

cancer ($n = 3$) and non-cancer ($n = 3$) animals were further evaluated. All isolates were confirmed to be *S. phocae* by sequence analysis of 16S rDNA. The *S. phocae* isolates were serologically Lancefield group F ($n = 3$) and G ($n = 3$), produced alkaline phosphatase and leucine arylamidase enzymatic activity and were all susceptible to bacitracin. Acid production was observed from starch and glycogen in five isolates (83%), from ribose in four isolates (67%), and one isolate exhibited alpha and beta-galactosidase activity. Although the sample size was small, there was equal distribution of Lancefield groups within cancer and non-cancer animals and between sexes.

Chlamydiaceae PCR was performed on DNA extracted from 59 wild, 28 stranded, and six carcinoma adult sea lions swabs. All samples were negative for *Chlamydia/Chlamydophila* spp. Swabs were also negative for *Brucella* by PCR analysis from 59 wild, 30 stranded, and five carcinoma adult sea lions.

4. Discussion

Bacterial growth and mild or no inflammation occurred in approximately 75% of these animals suggesting that most of the bacteria isolated are transient or commensal organisms. As observed in terrestrial mammalian species, bacterial colonization in California sea lions increased with age, both in the number of isolates per animal and diversity of bacteria species (Bara et al., 1993; Otero et al., 2000). Many of the bacteria identified in the wild sea lions are similar to vaginal flora isolated from other healthy terrestrial animals (Hirsh and Wiger, 1977; Doyle et al., 1991) and are comparable to bacteria isolated from a study of seven wild California sea lions (Johnston and Fung, 1971).

Some bacteria in the present study appear to be unique vaginal and preputial microflora in the California sea lion and many could not be identified to species level using standardized biochemical techniques. *P. phenylpyruvicus*, the most frequently isolated bacterium, is an inhabitant of marine ecosystems (Cavanagh et al., 1996) and is commonly isolated from fish (Gonzalez et al., 2000). The organism can cause invasive disease in humans (Guttigoli and Zaman, 2000) and has been isolated from lesions and tissues in

Antarctic fur seals (*Arctocephalus gazelle*) (Baker and McCann, 1989). The non-fermentative gram-negative bacilli group cultured from all age classes of wild sea lions of this study is generally considered avirulent and is often found in mixed cultures (Murray, 1999). Beta-hemolytic streptococci were cultured from each group of adult sea lions and are known to be commonly isolated from superficial abscesses, wounds, ocular and urethral discharges and umbilici of stranded California sea lions (Thornton et al., 1998). *Streptococcus phocae* is a beta-hemolytic streptococci that is considered a secondary and opportunistic pathogen, initially characterized in harbor seals (*Phoca vitulina*) and grey seals (*Haliochoerus grypus*) (Skaar et al., 1994), but has subsequently been reported in starving South African fur seals (Henton et al., 1999) and in harbor and grey seals in the North and Baltic seas (Vossen et al., 2004).

The Lancefield serology, biochemical profiles and bacitracin susceptibility of the *S. phocae* isolated from the California sea lions in the present study were generally consistent with previously reported *S. phocae* isolates except for their expression of Lancefield group G cell surface antigen. (Skaar et al., 1994; Henton et al., 1999; Vossen et al., 2004). *S. phocae* is not previously known to belong to Lancefield group G, except in southern sea otters (*Enhydra lutris nereis*) in California (Imai, unpublished data). Prior to our characterization of *S. phocae* in Lancefield group G, these isolates were erroneously identified using routine diagnostics as *S. canis*. The *S. phocae* isolates were differentiated from *S. canis* based on absence of arginine dihydrolase, alpha-galactosidase, beta-glucuronidase and beta-galactosidase activity, susceptibility to bacitracin (Hirsh and Biberstein, 2004) and 16S rDNA sequencing.

Many of the less frequently identified bacteria in this study such as *Edwardsiella tarda*, *Klebsiella pneumoniae*, *Arcanobacterium phocae*, and *Pseudomonas* spp. are often opportunistic pathogens or secondary invaders that have been previously isolated from tissues in diseased California sea lions (Sweeney and Gilmartin, 1974; Howard, 1983; Thornton et al., 1998; Johnson et al., 2003). The *Salmonella* spp. isolated from pups and adult females on San Miguel Island and from a female with urogenital carcinoma was not unexpected because salmonellosis has been previously reported in sea lions on San Miguel Island

(Gilmartin et al., 1979) and from stranded sea lions in rehabilitation (Thornton et al., 1998; Smith et al., 2002).

Animals in rehabilitation had significant shifts in their microbial flora away from the common bacteria isolated in the wild animals to coliform, environmental, and saprophytic microorganisms. This shift may have occurred due to changes in the immunologic status of the animals with illness, culture sampling after death, or possibly from fecal and environmental contamination of the vagina and prepuce while being confined in a cage or pen. The significant differences in bacterial growth detected in this study reinforce the notion that normal flora studies should be attempted only on apparently healthy free-ranging wild animals.

The urogenital carcinomas of sea lions were strongly associated with beta-hemolytic streptococci in the females suggesting the organism may play a role in the development of the disease; however, because no association was found in the males with urogenital carcinomas, there appears to be an interaction between sex and the prevalence of beta-hemolytic *Streptococcus* in animals with carcinomas. Except in carcinoma cases, beta-hemolytic streptococci were isolated from males more often than females, in both wild and stranded animals suggesting that the organism could be part of the commensal preputial microflora and possible sexually transmitted to females. Beta-hemolytic *Streptococcus* infection and associated inflammatory mediators may have a promotional effect on urogenital carcinogenesis in female California sea lions or the carcinomas may simply provide an altered environment favorable for beta-hemolytic streptococci growth.

The role of bacteria as a cofactor in the development of urogenital carcinomas in California sea lions remains unclear, yet continued research into the association with beta-hemolytic streptococci is warranted. The description of normal bacterial flora of the vagina and prepuce of California sea lions presented in this study should aid in future investigations of reproductive disorders of sea lions and the diagnosis of bacterial diseases in captive sea lions.

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