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## Geographical dissemination of *Leptospira interrogans* serovar Pomona during seasonal migration of California sea lions

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### ABSTRACT

Leptospirosis is one of the most widespread bacterial zoonoses in the world and affects most mammalian species. Although leptospirosis is well documented and characterized in terrestrial species, less information is available regarding the distribution and impact of leptospirosis in marine mammals. Additionally, the role of animal migrations on the geographical spread of leptospirosis has not been reported. Periodic epizootic outbreaks of acute leptospirosis among California sea lions (*Zalophus californianus*) have been reported since 1971. In this study, we collected samples from California sea lions stranded along the Pacific coast of North America during the most recent epidemic in 2004, and maintained leptospirosis surveillance of the California sea lion population along the California coast through 2007. Several isolates of *Leptospira interrogans* serovar Pomona were obtained from kidney and urine samples collected during this study, a finding consistent with serological evidence that California sea lions are persistently exposed to this leptospiral serovar. Combined, these data support a model whereby California sea lions are maintenance hosts for *L. interrogans* serovar Pomona, yet periodically undergo outbreaks of acute infection. During the 2004 outbreak, the incidence of new leptospirosis cases among California sea lions coincided with the seasonal movement of male sea lions from rookeries along the coast of central and southern California north as far as British Columbia. These data show that seasonal animal movement contributes to the distribution of leptospirosis across a large geographical region.

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### 1. Introduction

Pathogenic *Leptospira* cause leptospirosis, one of the most widespread bacterial zoonoses known. Leptospirosis

develops either into a chronic infection with low mortality or into an acute, potentially lethal infection (Faine et al., 1999). Bacteria disseminate via transient septicemia and localize in kidney and liver. Release of viable infectious bacteria to the surrounding environment during urination perpetuates disease transmission to naïve hosts (Faine et al., 1999).

Sporadic leptospirosis epizootic events among California sea lions (*Zalophus californianus*) provide an opportunity to

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study this disease in migratory marine mammals. This species breeds in rookeries along the southern California coast during May and June. Female sea lions usually remain near the rookeries to nurse pups or move north as far as Monterey Bay (Melin, 2002), while males travel north and spend the winter in a region extending from California to southern Alaska (Melin, 2002; Maniscalco et al., 2004). Although these animals congregate in rookeries and other haul-out sites along the coast, they periodically disperse to forage for food (Melin et al., 2008). Male California sea lions can travel up to 51 km/day and cover 644 km on a single foraging trip (Weise et al., 2006). California sea lions ability to travel over large distances gives rise to the potential geographical spread of zoonotic diseases like leptospirosis. Sick or injured animals either die at sea or are stranded on beaches; this leads to a situation that can contribute to disease transmission to terrestrial animals. In this report, we provide evidence that California sea lions are maintenance hosts for *Leptospira interrogans* serovar Pomona and that seasonal movement contributes to geographical spread of leptospirosis.

## 2. Materials and methods

### 2.1. Sample collection

Samples were obtained from animals stranded along the Pacific coast of North America, from San Luis Obispo County in California to Ucluelet, Vancouver Island, British Columbia. When possible, live animals were relocated to the Marine Mammal Center (Sausalito, CA) for clinical analysis and treatment, otherwise, urine samples were collected by either free flow capture, cystocentesis, or catheterization. Sera were collected from the caudal gluteal vein. Kidney and urine samples were collected from animals that died during treatment or found dead on the beach during necropsy, and either frozen immediately at  $-80^{\circ}\text{C}$  or processed for bacterial culture.

### 2.2. Clinical analysis

Clinical signs leading to a positive diagnosis of leptospirosis included dehydration, polydipsia, depression or emaciation. Evidence for leptospirosis during gross necropsy included the appearance of swollen kidneys, pale tan renal cortices, or subcapsular renal hemorrhages. Serum samples were evaluated for blood urea nitrogen (BUN), creatinine, sodium, phosphorus, and calcium levels. The following criteria were used as indicators of leptospirosis: BUN  $>100$  mg/dl, creatinine  $>2$  mg/dl, sodium  $>155$  meq/l, and phosphorus  $>$  calcium (Gulland et al., 1996; Colagross-Schouten et al., 2002).

### 2.3. Fluorescent antibody analysis

Direct immunofluorescence was used to detect *Leptospira* in urine and kidney homogenates as described previously (Wagenaar et al., 2000). Briefly, samples were placed on glass slides and fixed with acetone followed by staining with fluorescein isothiocyanate (FITC)-labeled rabbit anti-*Leptospira* conjugate. The conjugated antisera

was prepared in-house following a protocol described elsewhere (Ellis et al., 1982). Slides were rinsed with phosphate buffered saline (PBS), counter stained with Flazo Orange (obtained from Mark Wilson, National Veterinary Services Laboratory, Ames, IA, USA) and examined using a Nikon Eclipse E800 fluorescence microscope fitted with a B-2E/C filter (Bolin et al., 1989; Zuerner, 2006).

Indirect immunofluorescent analysis was used to detect *Leptospira* in samples of formalin-fixed, paraffin-embedded tissue (PEFF) as described previously (Matsunaga et al., 2006). Briefly,  $4\ \mu\text{m}$ -thick sections of PEFF tissue on glass slides were deparaffinized with xylenes and ethanol using standard procedures (Sheehan and Hrapchak, 1980). Tissue sections were blocked with 10% normal horse sera, then stained overnight at  $4^{\circ}\text{C}$  with rabbit anti-LipL21 (Cullen et al., 2003) or anti-LipL32 (Haake et al., 2000) sera (generously provided by P. Cullen and D. Haake, respectively). Samples were washed with PBS before incubation with goat anti-rabbit Alexa 488-conjugated secondary antibody (Molecular Probes) for 1 h in the dark at room temperature. Samples were washed with PBS, counter stained with DAPI (4',6-diamidino-2-phenylindole, 1.5 mg/ml), and mounted with SlowFade Light antifade (Molecular Probes). Images were captured with a Spot RT color CCD camera (Diagnostic Instruments, Inc.) mounted on a Nikon Eclipse E800 microscope using a  $100\times$  Pan Fluor objective, and B-2A and UV-2E/C filters, then merged using Spot Advanced Software (Diagnostic Instruments, Inc.).

### 2.4. Serology

Sera collected from stranded California sea lions were tested for the presence of antibodies to *Leptospira* using the microscopic agglutination test (MAT) (Ryu, 1970; Cole et al., 1973) as previously described (Colagross-Schouten et al., 2002). Doubling dilutions of sera were incubated with *L. interrogans* reference cultures for serovars Bratislava, Canicola, Hardjo, Icterohaemorrhagiae, and Pomona, and *Leptospira kirschneri* serovar Grippotyphosa. A positive agglutination titer cut-off for a single serum sample was  $\geq 1:400$ .

### 2.5. PCR analysis of urine and tissue samples

Detection of *Leptospira* DNA in urine and kidney samples was done using PCR. Genomic DNA was prepared using DNeasy kits (Qiagen) following the manufacturer instructions. Primers used to detect *Leptospira* sequences in clinical samples were directed to: (1) *L. interrogans* IS1500 sequences using primers P1 (5'-TTCGATTCAAAG-CATGGCTAACG-3') and M16 (5'-AAAGAAGGACTCAGC-GACTGCG-3') (Zuerner and Bolin, 1997); (2) *Leptospira borgpetersenii* IS1533 sequences using primers 1805 (5'-GCGGAAAGTGAACCGTATCGAG-3') and 1809 (5'-CTGATATTTCGCGGTTGGAAGG-3') (Zuerner, 1994; Zuerner et al., 1995); and (3) *L. kirschneri* flagella sequences using primers B64-I (5'-ACTAACTGAGAACTTCTAC-3') and B64-II (5'-TCCTTAAGTCGAACCTATGA-3') (Gravekamp et al., 1993). PCR amplification parameters were  $94^{\circ}\text{C}$  for 15 s,  $60^{\circ}\text{C}$  for 30 s, and  $65^{\circ}\text{C}$  for 1.5 min, for 50 cycles. Sample

dye was added to each sample and the entire reaction volume (50  $\mu$ l) applied to 1.5% agarose (Nusieve:GTG agarose, 3:1 ratio) and the products separated by electrophoresis, stained with ethidium bromide, and the reaction products visualized by ultraviolet illumination as described previously (Zuerner and Bolin, 1990).

## 2.6. Bacteriological culture and analysis

Tissue and urine samples from stranded animals were used to inoculate EMJH medium (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967) supplemented with 10% heat inactivated rabbit sera and transport medium (Zuerner, 2006) prior to overnight shipping to the National Animal Disease Center (NADC) for isolation. Upon receipt, samples were examined for the presence of spirochetes and contamination. If contaminating microorganisms were detected, samples were passed through 0.22  $\mu$ m bacteriological filters which retain most microorganisms, but which fail to block passage of *Leptospira*. The resulting filtrates were inoculated into fresh EMJH media. Cultures were incubated at 29 °C for up to 6 months and received periodic examination before being considered negative for *Leptospira*.

Genomic DNA was isolated from bacterial isolates using proteinase K digestion followed by phenol:chloroform extraction using standard techniques (Sambrook et al., 1989), then analyzed by restriction endonuclease analysis as described (Thiermann et al., 1985). Approximately 10  $\mu$ g of genomic DNA was digested with EcoRI, separated by agarose gel electrophoresis in 1% agarose gels, and the products detected by UV-illumination of ethidium bromide stained gels as described above.

## 3. Results

### 3.1. Characterization of the 2004 leptospirosis outbreak

During the 2004 outbreak of leptospirosis among California sea lions, the incidence of stranded animals increased between May and June as compared to the first 4 months of that year. Throughout the remainder of 2004, stranded animals were retrieved from along the Pacific coast across a region extending from San Luis Obispo County in central California to Ucluelet, Vancouver Island, British Columbia (Table 1). Five hundred sixty eight stranded California sea lions were identified, with most of the animals initially recovered alive. However, 8% of the animals were recovered either as carcasses or died before sample collection was possible, including several animal carcasses collected in northern latitudes that had frozen or undergone decomposition. In addition, several animals, including all animals identified in Oregon, were initially found on the beach, but returned to the water, with or without assistance, before samples could be collected for PCR or culture.

Eighty-seven percent ( $n = 496$ ) of the 568 stranded animals were evaluated for leptospirosis (Table 2). A positive diagnosis for leptospirosis was based on clinical evaluation and/or positive results using bacterial culture, PCR, detection of characteristic lesions during necropsy

**Table 1**

Recovery of stranded California sea lions by state or province during 2004.

Month	No. of animals stranded			
	California	Oregon	Washington	British Columbia
January	9	0	0	0
February	7	0	0	0
March	7	0	0	0
April	5	0	0	0
May	15	0	0	0
June	21	0	0	0
July	57	0	0	0
August	95	7	3	0
September	116	18	15	3
October	84	0	22	7
November	35	0	11	3
December	22	0	6	0
Total	473	25	57	13

**Table 2**

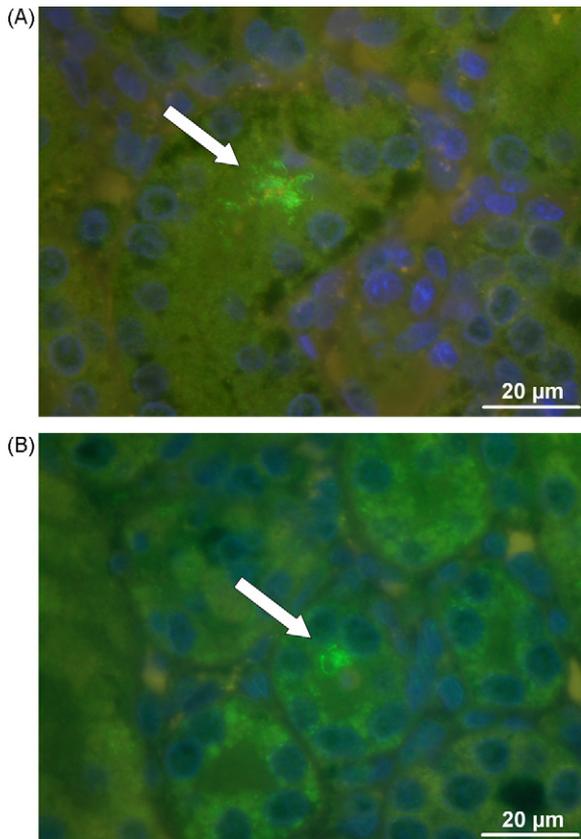
Leptospirosis prevalence during the 2004 epidemic.

Month	No. of positive diagnosis (no. of evaluated)	
	California	Washington
January	2 (9)	0 (0)
February	0 (7)	0 (0)
March	1 (7)	0 (0)
April	0 (5)	0 (0)
May	0 (15)	0 (0)
June	4 (21)	0 (0)
July	22 (57)	0 (0)
August	73 (95)	1 (1)
September	106 (116)	3 (4)
October	62 (84)	8 (13)
November	28 (35)	2 (2)
December	11 (22)	0 (0)
Total	309 (473)	14 (22)

No samples were collected from animals in Oregon. One sample was tested (positive) from animals collected in British Columbia.

and microscopic analysis of tissue and urine samples. Where possible, sera were collected and tested for the presence of *Leptospira*-agglutinating antibodies using the MAT, and examined for increased blood urea nitrogen (BUN), creatinine, and an inverted calcium:phosphorous ratio (Colagross-Schouten et al., 2002). Upon necropsy, swelling of the kidneys with loss of renule definition was considered characteristic of leptospirosis (Gulland et al., 1996). Histological analysis confirmed these animals exhibited tubulointerstitial nephritis, and the presence of *Leptospira* in kidney tissue was confirmed using indirect immunofluorescence microscopy (Fig. 1) or immunohistochemistry (data not shown). Sixty-five percent ( $n = 324$ ) of the 496 animals evaluated were diagnosed with leptospirosis (Table 2). The proportion of stranded California sea lions with leptospirosis recovered along the California coast was 65.3%, with the highest rate of infection (100%) occurring in Mendocino county and the lowest rate of infection (20%) in San Luis Obispo county, the northernmost and southernmost counties, respectively (Table 3).

California sea lions with confirmed cases of leptospirosis along the California coast during the 2004 epidemic



**Fig. 1.** Detection of *Leptospira* in kidney tissue by immunofluorescence. Immunofluorescent analysis of PEFF tissue sections from a California sea lion kidney reveals the presence of the *Leptospira* using antisera directed against LipL21 (A) and LipL32 (B). The bar in each image provides a 20 µm reference marker. *Leptospira* are stained bright green (arrow) and the nuclei of host cells are stained blue.

**Table 3**  
2004 outbreak: recovery of stranded California sea lions by county.

County	Leptospirosis no. (% total)	Total
Mendocino	14 (100)	14
Sonoma	22 (79)	28
Marin	41 (79)	52
Contra Costa	4 (67)	6
Alameda	7 (100)	7
San Francisco	30 (81)	37
San Mateo	30 (71)	42
Santa Cruz	56 (73)	77
Monterey	88 (71)	124
San Luis Obispo	17 (20)	86
Total	309 (65)	473

Counties are sorted geographically with the northernmost county at the top.

had a mortality rate of 73%. Most infected California sea lions were juveniles (71%), with nearly equal proportions of adult/subadult (14%) and yearling (15%) infections. Ninety-two percent of the infected California sea lions were male; consistent with the vast majority of animals that disperse northward being male.

### 3.2. Disease surveillance

After the 2004 epidemic, surveillance and disease assessment of stranded California sea lions retrieved along the California coast continued through 2007. No strandings consistent with leptospirosis were detected in 2005. In 2006, two stranded California sea lions were retrieved and were positive for leptospirosis. In 2007, forty-nine stranded California sea lions were retrieved; 63% ( $n = 31$ ) of these animals were diagnosed with leptospirosis. Seventy-four percent ( $n = 23$ ) of the animals with leptospirosis identified in 2007, and 100% ( $n = 2$ ) of the leptospirosis-infected animals in 2006, were either euthanized or died during treatment. During 2007, 58% ( $n = 18$ ) of the animals diagnosed with leptospirosis were juveniles, with the balance of infected animals classified as adult or subadult.

### 3.3. Bacterial and serological analysis

Urine and kidney tissue represent the most likely sources of material from which pathogenic *Leptospira* can be obtained, because the bacteria reside primarily in the convoluted tubules of the kidney, are passed to the bladder, and released to the outside environment during urination. PCR analysis of California sea lion urine and tissue samples detected the presence of *L. interrogans* IS1500 sequences (Zuerner and Bolin, 1997). Selected samples analyzed with species-specific PCR primers failed to detect the presence of either *L. borgpetersenii* or *L. kirschneri* DNA.

Serum samples from California sea lions were tested for antibodies directed against *Leptospira* serovars Bratislava, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona using the MAT. Positive sera often agglutinate multiple *Leptospira* serovars at different titers due to shared epitopes, and this occurred throughout samples analyzed in the present study. Antibody titers from animals collected during both the 2004 leptospirosis outbreak and subsequent surveillance in 2007 were highest to serovar Pomona. Additionally, 25% (7/28) of stranded California sea lions that had no clinical signs of leptospirosis and were tested by the MAT also had positive leptospiral antibody titers.

Bacteriological isolates were obtained from eight California sea lions from 2004 through 2007. Total genomic DNA was isolated from each isolate and characterized using restriction endonuclease analysis using EcoRI, a method shown previously to be useful for characterizing *L. interrogans* serovars (Thiermann et al., 1985). All eight isolates were identified as *L. interrogans* serovar Pomona (data not shown), a finding consistent with both PCR and serological data.

## 4. Discussion

This study provides evidence that *L. interrogans* serovar Pomona infections persist among California sea lion populations, and that movement of infected California sea lions can facilitate spread of this disease over a large geographical region. Periodic outbreaks of leptospirosis

along the Pacific coast of North America among California sea lions have been reported since 1971 (McIlhattan et al., 1971; Dierauf et al., 1985; Gulland et al., 1996; Godinez et al., 1999; Colagross-Schouten et al., 2002; Acevedo-Whitehouse et al., 2003; Greig et al., 2005; Cameron et al., 2008), with the most recent outbreak in 2004 being the primary focus of this study.

Data obtained during this study are consistent with previous findings (Dierauf et al., 1985; Gulland et al., 1996; Greig et al., 2005) that show *L. interrogans* serovar Pomona is the primary cause of leptospirosis in California sea lions. The results of serological, bacteriological, and PCR analyses conducted during this study provide evidence that *L. interrogans*, but neither *L. borgpetersenii* nor *L. kirschneri* are associated with disease in California sea lions. The lack of detectable *L. kirschneri* DNA and presence of low serovar Grippotyphosa antibody titers is significant. Elephant seals (*Mirounga angustirostris*) can be infected with *L. kirschneri* serovar Grippotyphosa (Cameron et al., 2008), and the range of these marine mammals overlaps the range of California sea lions. Furthermore, while potential transmission of *L. interrogans* serovar Pomona from California sea lions to elephant seals during captivity may occur (Colegrove et al., 2005), there is currently no evidence for *L. kirschneri* transmission from elephant seals to sea lions.

California sea lions likely serve as maintenance hosts for *L. interrogans* serovar Pomona. Twenty-five percent of stranded animals with no clinical evidence for leptospirosis tested positive for anti-*Leptospira* antibodies, a finding consistent with a recent study (Lloyd-Smith et al., 2007) that clearly shows California sea lions are persistently exposed to serovar Pomona. Juvenile California sea lions are most commonly found with clinical disease during epidemics. The severity of leptospirosis during periodic epizootics may involve the interplay of several factors including infection of younger animals unable to mount sufficient protective immunity to limit disease manifestation (Gulland et al., 1996), malnutrition, and exposure to toxins that impair immunological maintenance of chronic infection (Gilmartin et al., 1976). The possibility that exposure to *Leptospira*-contaminated urine while on shore or while swimming in fresh water estuaries may cause leptospirosis in California sea lions cannot be excluded. Male California sea lions spend between approximately 20% and 55% of their time resting on shore (Weise et al., 2006), providing time for exposure to terrestrial mammals chronically infected with pathogenic *Leptospira*. However, if this alternative route of exposure were a significant source of infection then we would expect to find serological, bacteriological, or PCR evidence for exposure to multiple *Leptospira* species and/or serovars. To date, all leptospiral isolates obtained from California sea lions including those from 1971 and 1988 are *L. interrogans* serovar Pomona, and the predominant serological response is to serovar Pomona.

An important novel finding of this study is that California sea lions with leptospirosis can disperse over large geographical regions. The coastal terrain along which California sea lions travel is rugged and offers limited access for detecting or recovering stranded animals. Despite bias induced by limited access to the coastline,

it is apparent that the incidence of stranded animals changed over time in different geographical regions consistent with dispersal of male California sea lions from southern California to British Columbia (Table 1). In Mendocino county (the northernmost county sampled from California) 100% of the sea lions tested were diagnosed with leptospirosis, and were recovered later in the year than those recovered from San Luis Obispo county (the southernmost county sampled in California), where the leptospirosis rate was 20%. Additionally, the number of stranded animals peaked in Washington and British Columbia in October (Table 1), a month later than the statewide peaks in California or Oregon. Leptospirosis has a latency period of 10–14 days between contact and development of clinical signs of infection; we predict that animals are able to travel during this latency period. *Leptospira* are not salt tolerant, suggesting that infection of naïve animals likely occurs through close contact with infected animals at haul-out sites on shore. By maintaining a portion of the population in a latent disease state, the distribution of infected animals is perpetuated as animals disperse across a broad geographical area. Infected animals were often found near fresh water estuaries, potentially increasing the likelihood of disease transmission to humans, domestic animals and terrestrial wildlife, and therefore leptospirosis-infected California sea lions pose a potential public health threat.

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