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Antibiotic Efficacy in Eliminating Leptospiruria in California Sea Lions (*Zalophus californianus*) Stranding with Leptospirosis

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Abstract

Stranded California sea lions (*Zalophus californianus*) along the California coast have been diagnosed with leptospirosis every year since at least the 1980s. Between September 2010 and November 2011, we followed 14 stranded California sea lions that survived to release and evaluated antibiotic efficacy in eliminating leptospiruria (urinary shedding of leptospires). Leptospiruria was assessed by real-time PCR of urine and urine culture, with persistence assessed using longitudinally collected samples. Serum chemistry was used to assess recovery of normal renal function. Microscopic agglutination testing (MAT) was performed to assess serum anti-*Leptospira* antibody titers, and the MAT reactivity patterns were consistent with *L. interrogans* serovar Pomona infection frequently observed in this population. Animals were initially treated for 6 to 16 d (median = 10.5; mean = 10.8) with antibiotics from the penicillin family, with some receiving additional antibiotics to treat other medical conditions. All urine cultures were negative; therefore, the presence of leptospiruria was assessed using PCR. Leptospiruria continued beyond the initial course of penicillin family antibiotics in 13 of the 14 sea lions, beyond the last antibiotic dose in 11 of the 14 sea lions, beyond recovery of renal function in 13 of the 14 sea lions, and persisted for at least 8 to 86 d (median = 45; mean = 46.8). Five animals were released with no negative urine PCR results detected; thus, their total shedding duration may have been longer. Cessation of leptospiruria was more likely in animals that received antibiotics for a greater duration, especially if coverage was uninterrupted. Real-time PCR results indicate that an antibiotic protocol commonly used to treat leptospirosis in rehabilitating California sea lions does not eliminate leptospiruria. It is possible that antibiotic protocols given for a longer duration and/or including other antibiotics may be effective in eliminating leptospiruria. These results may have important human and animal health implications, especially in rehabilitation facilities, as *Leptospira* transmission may occur through contact with animals with persistent leptospiruria.

Key Words: antibiotic, California sea lion, *Zalophus californianus*, *Leptospira interrogans*, leptospiruria, renal disease, chronic shedding

Introduction

Leptospirosis is a zoonosis caused by pathogenic species in the spirochete genus *Leptospira*, and various *Leptospira* spp. cause significant morbidity and mortality worldwide in wildlife, domestic animals, and humans (Faine et al., 1999). Pathogenic *Leptospira* spp. colonize the kidneys of infected individuals and are shed in the urine (leptospiruria); for most serovars and most host species, leptospiruria is the main mechanism of transmission to others via direct contact or environmental contamination (Faine et al., 1999). Leptospirosis was first reported in California sea lions (*Zalophus californianus*) in autumn of 1970 during an epizootic that caused widespread stranding and mortality on the California and Oregon coasts (McIlhattan et al., 1971; Vedros et al., 1971). The causative organism in this outbreak, which was identified from all leptospiral isolates obtained subsequently from wild California sea lions, was *L. interrogans* serovar

Pomona (McIlhattan et al., 1971). Further analysis based on variable number tandem repeat typing showed that all isolates from California sea lions, spanning over three decades, have belonged to a distinctive clade not associated with any other host species (Zuerner & Alt, 2009). Since the early 1980s, The Marine Mammal Center (TMMC) has treated California sea lions stranding along the California coast during recurrent epizootics of leptospirosis, and the disease has been a dominant cause of sea lion strandings during this period (Gerber et al., 1993; Greig et al., 2005).

At TMMC, diagnosis of leptospirosis is based on typical clinical signs and serum chemistry abnormalities (Gulland et al., 1996; Colagross-Schouten et al., 2002; Greig et al., 2005), and treatment consists of supportive care and antibiotics. Due to the severity of the renal disease, roughly two-thirds of sea lions exhibiting signs of leptospirosis do not survive (Gulland et al., 1996), but those that recover are released back into the wild. Antibiotic therapy for leptospirosis in California sea lions is guided by protocols used in other species (Greene et al., 1998; Alt et al., 2001; Sykes et al., 2011) and primarily involves a 10 to 14 d course of antibiotics in the penicillin family. Antibiotics within the tetracycline family are sometimes administered in addition to or instead of antibiotics in the penicillin family. The effect of antibiotic therapy on infection status in California sea lions is unknown as neither urine nor kidney samples are routinely collected to assess leptospiruria or renal leptospire carriage.

During their rehabilitation and treatment at TMMC, we followed 14 California sea lions that stranded with leptospirosis between September 2010 and November 2011 and survived to release. The goal of this study was to determine the duration of leptospiruria during rehabilitation and to assess whether leptospiruria continued beyond termination of antibiotic therapy and return of normal renal function. These results are important for assessing antibiotic efficacy in eliminating leptospiruria, for guiding future treatment of California sea lions stranding with leptospirosis, and for assessing the infectious risk that these animals pose to other animals, including humans, both during rehabilitation and upon release. These results are also important from a broader ecological perspective as they lend insight into the potential for chronic carriage—a proposed mechanism for long-term persistence of the pathogen in the California sea lion population.

Methods

Case Selection

Thirty-five California sea lions that stranded and were admitted to TMMC between 1 September 2010 and 30 November 2011 were diagnosed with leptospirosis based on abnormal serum chemistry values consistent with leptospirosis-induced renal impairment: BUN ≥ 100 mg/dl and creatinine ≥ 2 mg/dl (Greig et al., 2005). Fourteen of these animals survived to release and were included in our analyses of antibiotic efficacy in eliminating leptospiruria. Samples for this study were collected from California sea lions during their routine clinical care at TMMC and under the approved National Oceanic and Atmospheric Administration, National Marine Fisheries Service–Southwest Region Stranding Agreement.

Antibiotic Treatment

Antibiotic treatment was initiated within 72 h of admission. All animals were treated for 6 to 16 d with antibiotics in the penicillin family (30,000 units/kg penicillin G IM q 48 h when inappetant and 22 mg/kg amoxicillin tetrahydrate PO q 12 h when eating), and some were given additional antibiotics during the course of rehabilitation to treat other medical conditions (Figure 1). These additional antibiotics included doxycycline (5 to 10 mg/kg PO q 12 h), oxytetracycline (20 to 40 mg/kg IM q 48 h), ciprofloxacin (5 to 10 mg/kg PO q 12 h), enrofloxacin (5 to 10 mg/kg IM q 24 h), and ceftiofur (6.6 mg/kg IM once). The duration of antibiotic coverage varied among individuals, and for the purposes of analysis it was calculated for each animal in three different ways: (1) total number of days covered by any antibiotic or antibiotic combination, (2) maximum number of consecutive days covered by any antibiotic or antibiotic combination, and (3) maximum number of consecutive days covered during initial treatment with penicillin family antibiotics. For all methods, "coverage" was determined by the recommended dosage interval—for example, a single dose of an antibiotic with a dosing regimen of once every 48 h was calculated as covering an animal for 48 h. When indicated, animals received the following supportive care and treatments: subcutaneous fluid administration, gastrointestinal protectants (e.g., famotidine, kaolin pectin), analgesics (e.g., carprofen, ketoprofen, tramadol), and antiparasitics (e.g., ivermectin).

Sample Collection, Processing, and Testing

Urine and serum were collected approximately every 14 d (range of collection intervals for urine = 9 to 44, median = 14; serum range = 1 to 21, median = 14) until release, which occurred between 32 and

Figure 1. Clinical course and treatment history of each animal, showing the days on which antibiotic treatment was received, days on which urine was collected for PCR and culture, and the day on which BUN and serum creatinine returned to within the normal range (BUN ≤ 83 mg/dl and creatinine ≤ 1.1 mg/dl). Results of urine PCR are marked as P (positive) or N (negative). If the sample was also cultured, the box is shaded gray. The day that both BUN and serum creatinine concentrations have returned to within the normal range is indicated by an "X." The dashed lines indicate the beginning and the end of the 9th wk since admission. *The first two PCR positive results for animal 9918 were collected from a holding container in which the animal had been placed specifically for urine collection. The container had been steam washed and bleached prior to its use for collecting urine; therefore, although not aseptically collected, contamination of the urine was unlikely.

93 d after admission to TMMC (median = 62.5 d). While under anesthesia, urine was collected using a sterile technique by cystocentesis or catheterization (n = 50) and by free catch (n = 1). Urine was also collected from one animal while awake and contained in a sterilized holding cage (n = 2). The first blood sample was collected between 1 and 5 d of admission (median = 3). Blood was collected

from the caudal gluteal vein, allowed to clot, centrifuged at 3,000 G, and the serum separated. Serum chemistry analyses were performed within 24 h. The remaining serum was divided into aliquots and frozen at -80° C until serum microscopic agglutination testing (MAT) could be performed. Banked aliquots for MAT from this first sample were available for all but one animal. For this animal, the first sample for MAT was collected 18 d after admission.

To confirm the diagnosis of leptospirosis and to assess antibiotic efficacy in eliminating leptospiruria, real-time PCR testing (all samples; $n = 53$) and culture ($n = 29$) of urine samples were performed to detect leptospiral DNA (PCR) or *Leptospira* spp. (culture). Sample processing, PCR, and culture were as described for wild-caught California sea lions in Prager et al. (2013). With the exception of one animal (9830) from which urine was collected the same day antibiotics were begun, urine sample collection occurred after antibiotics had been initiated and started between 3 and 31 d of admission (median = 15 d). The real-time PCR assay used was specific for pathogenic *Leptospira* spp. (Wu et al., 2014). Leptospiruria was considered eliminated if an individual had negative results for both PCR and culture (if both were done) or just PCR (if not cultured) on two consecutive urine samples collected at least 7 d apart. Serum chemistry analysis was used to diagnose leptospirosis and to assess recovery of renal function. Leptospirosis was diagnosed if BUN ≥ 100 mg/dl and creatinine ≥ 2 mg/dl (Greig et al., 2005) in the absence of clinical signs indicative of other diseases that can cause similar elevations in BUN and creatinine such as amyloidosis or urogenital carcinoma. BUN ≤ 83 mg/dl and creatinine ≤ 1.1 mg/dl were considered within the normal range for a healthy sea lion (Roletto, 1993; Prager et al., 2013) and, thus, indicative of a return to normal renal function. Serum MAT was performed at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, using live-cultured antigen against 19 *Leptospira* serovars representing 17 serogroups (*L. interrogans* serovars Australis, Autumnalis, Bratislava, Bataviae, Canicola, Djasiman, Grippotyphosa, Icterohaemorrhagiae, Mankarso, Pomona, Pyrogenes, and Wolffi; *L. borgpetersenii* serovars Ballum, Javanica, and Tarassovi; *L. weilii* serovar Celledoni strain Celledoni; *L. kirschneri* serovar Cynopteri strain 3522C; and *L. santarosai* serovars Borincana and Georgia). Sera were tested at doubling dilutions starting from 1:100, and agglutination was read using darkfield microscopy. The endpoint titer was reported as the highest dilution that agglutinated at least 50% of the cells for each strain tested (Dikken & Kmety, 1978).

Statistical Analysis

Fourteen California sea lions were included in the study; however, the time to release varied among individuals from 5 to 14 wks, causing right censoring of the data. To adjust for this in our statistical analyses, we only included animals that were released during or after Week 9 ($n = 9$). Week 9 was chosen because all four animals for which leptospiruria was considered eliminated had negative urine PCR results by then. The duration of antibiotic administration was calculated for the period between admission and the end of Week 9 (Day 63). Welch two sample *t*-tests were run in the "stats" package in *R* (R Development Core Team, 2013) to assess whether the mean duration of antibiotic coverage (using each of the three methods to calculate duration) differed between animals that were urine PCR positive at release and those that were negative. The Welch *t*-test was chosen to allow for the possibility of unequal variances among samples; other assumptions of the *t*-test were met. Fisher's exact test was run in the "stats" package in *R* to assess whether the probability of shedding at release differed between animals that received additional antibiotics (i.e., antibiotics from families other than the penicillin family) vs those that did not. Because tetracycline family antibiotics (doxycycline and oxytetracycline) were the most common additional antibiotics administered, Fisher's exact test was also run to assess whether the probability of shedding at release differed between animals that received an antibiotic in the tetracycline family ($n = 6$) and those that did not ($n = 3$). Gastrointestinal (GI) protectants may interfere with antibiotic absorption; therefore, Fisher's exact test was run to assess whether the probability of shedding at release differed between animals that received GI protectants ($n = 6$) vs those that did not ($n = 3$).

Results

PCR results revealed urinary shedding of *Leptospira* spp. DNA (henceforth "leptospiruria") at some point during rehabilitation in all 14 California sea lions, and indicated that the minimum duration of leptospiruria ranged from 8 to 86 d (median = 45; mean = 46.8; Figure 1). Leptospiruria was considered to be eliminated in 4 of 14 sea lions (range = 8 to 47 d between admission and the last PCR positive urine; median = 38; mean = 33; Figure 1), but the remaining 10 animals were still PCR positive on the last sample prior to release. Therefore, calculations of the duration of leptospiruria in these animals (range = 29 to 86 d between admission and the last PCR positive urine; median = 51; mean = 52.5; Figure 1) may underestimate the true duration.

Leptospiruria was detected in all but one California sea lion (13 of 14) after the last day of their initial course of antibiotics in the penicillin

family (range = 19 to 76 d after the last dose; median = 31; mean = 37.2; Figure 1). The exception was a California sea lion that had positive PCR results during its initial penicillin antibiotic treatment, but for which urine PCR results were not available again until 35 d after the last dose of antibiotics was given (animal 9912 in Figure 1). Leptospiruria was detected in 11 of the 14 sea lions beyond the last dose of any antibiotics (range = 8 to 62 d; median = 31; mean = 32.6; Figure 1).

Leptospiruria was detected in 13 of the 14 California sea lions either at the time of, or beyond, return to normal renal function as determined by a decline into, and maintenance within, the normal range of serum BUN and creatinine concentrations (range = 0 to 54 d after return to normal renal function; median = 17; mean = 23.8; Figure 1). One animal did not fit this pattern (animal 9999 in Figure 1); serum BUN and creatinine concentrations declined into the normal range 15 d after the last positive PCR result.

The nine California sea lions that were released during or after Week 9 were included in statistical analyses to test whether antibiotic treatment history was associated with continued leptospiruria at release. Animals that were no longer shedding leptospires at release had, on average, been given antibiotics for longer, both when examining total days on any antibiotics and total consecutive days on any antibiotics (Figure 2a & 2b; Table 1). However, there was no difference in the duration of initial treatment with antibiotics in the penicillin family for animals that were not shedding vs shedding at release (Table 1). Intriguingly, all animals that were no longer shedding at release had

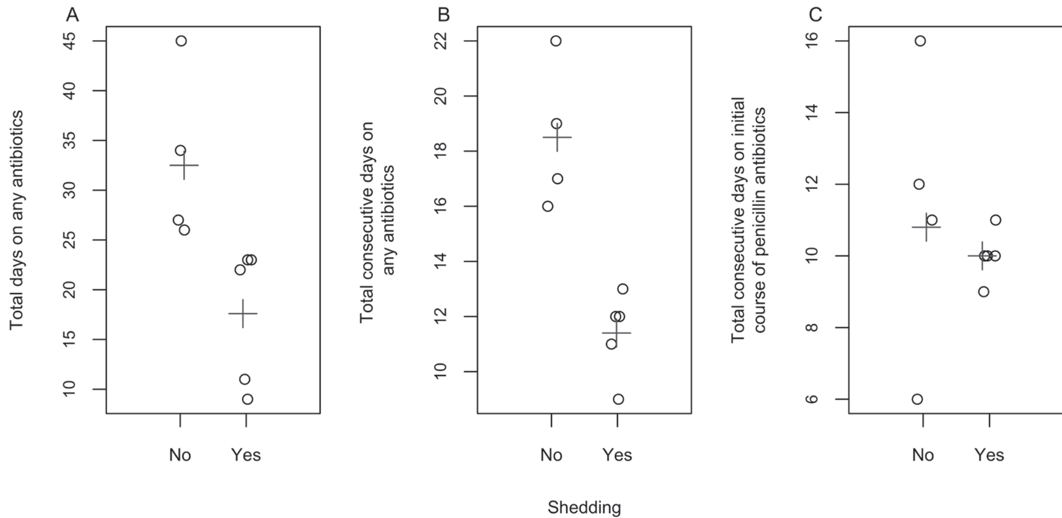

Figure 2. Number of days (y-axis) that antibiotics were received for animals released during or after Week 9 that were not shedding (No; $n = 4$) or were shedding (Yes; $n = 5$) at release; the duration of treatment is calculated based on (A) the total number of days on any antibiotic, (B) the total consecutive number of days on any antibiotic, and (C) the total consecutive number of days of the initial antibiotic course within the penicillin family. Open circles represent the number of days of treatment for each individual, and crosses represent the mean number of days of treatment for that group (shedding or not shedding).

Table 1. Results of Welch two sample *t*-tests comparing the mean number of days of antibiotic therapy in California sea lions (*Zalophus californianus*) that were not shedding (NS) and shedding (S) at the time of release; sample size for NS was four animals, and sample size for S was five animals for the first three antibiotic protocols and two animals for the tetracycline protocols. Degrees of freedom (*df*; approximated using the Welch-Satterthwaite equation [Satterthwaite, 1946; Welch, 1947]), and 95% confidence intervals (95% CI) of the difference between means are reported.

Antibiotic protocol	NS	S	Mean NS	Mean S	T	*df*	*p* value	(95% CI)
Any antibiotic	4	5	33.0	17.6	2.9	5.7	0.03	(2.1 to 28.7)
Consecutive any antibiotic	4	5	18.5	11.4	4.8	4.5	< 0.01	(3.2 to 11.0)
Initial penicillin treatment	4	5	11.3	10.0	0.6	3.1	0.59	(-5.2 to 7.7)
Tetracycline	4	2	17.5	17.5	0.0	3.9	1.00	(-22.0 to 22.0)
Consecutive tetracycline	4	2	13.25	12.0	0.32	3.4	0.77	(-10.3 to 12.8)

received antibiotics in the tetracycline family, and three of the four had received other antibiotics in addition to the initial penicillin/amoxicillin antibiotics and the course(s) of tetracycline antibiotics. However, there was no significant difference in shedding probability detected between animals that received tetracycline family antibiotics (shedding, $n = 2$; not shedding, $n = 4$) vs those that did not (shedding, $n = 3$; not shedding, $n = 0$; Fisher's Exact Test for Count Data p value = 0.17), nor was time treated with a tetracycline antibiotic a significant predictor of shedding (total days as well as total consecutive days on a tetracycline antibiotic; Table 1). There was also no significant difference in shedding probability detected between animals that received additional non-penicillin antibiotics (shedding, $n = 3$; not shedding, $n = 4$) vs those that did not (shedding, $n = 2$; not shedding, $n = 0$; Fisher's Exact Test for Count Data p value = 0.44) or in animals that received GI protectants (shedding, $n = 2$; not shedding, $n = 4$) vs those that did not (shedding, $n = 2$; not shedding, $n = 1$; Fisher's Exact Test for Count Data $p = 0.52$). All urine cultures were either contaminated ($n = 2$) or had no growth ($n = 27$) by 6 mo and were thus considered negative.

MAT results from the first serum sample available indicated that all sea lions had serum antibodies that bound strongly to *L. interrogans* serovar Pomona antigen. Cross-reactivity was present in all samples as is typical for *Leptospira* MAT results; however, in 13 of the 14 sea lions, the highest titers were against *L. interrogans* serovar Pomona and ranged from 1:51,200 to 1:409,600. Four of these sea lions had equivalently high titers against one or both of *L. interrogans* serovars Bratislava and Autumnalis. For one animal, the highest antibody titer was against *L. interrogans* serovar Bratislava, with titers against *L. interrogans* serovars Pomona, Autumnalis, and Djasiman being one dilution lower. Three or more MAT results were available for each animal, with the time between the first and last sample ranging from 27 to 86 d. Rising titers were not detected in any animal as is typical for California sea lions that have stranded due to clinical impacts of leptospirosis (Prager, unpub. data).

Discussion

PCR results confirmed urinary shedding of DNA from pathogenic *Leptospira* spp. in all 14 California sea lions that were diagnosed with leptospirosis at admission by clinical signs and serum chemistry results. This leptospiruria persisted beyond the last antibiotic dose in 11 of the 14 animals; up until or beyond the return of renal function in 13 of the 14 animals; and until release in 10 of the 14 animals. These results indicate that leptospiruria was not eliminated by the standard antibiotic protocol used at TMMC during the period of this study (6 to 14 d of 30,000 units/kg penicillin G IM q 48 h when inappetant and 22 mg/kg amoxicillin tetrahydrate PO q 12 h when eating). We also found that elimination of leptospiruria does not occur at the same time as return of normal renal function; therefore, continued carriage and shedding must be assessed by detection of the organism via PCR or culture, not by presence of clinical signs. In addition, leptospiruria was detected for at least 86 d, and asymptomatic leptospiruria was detected for at least 54 d, indicating that chronic and asymptomatic carriage of this pathogen are possible in California sea lions. An ancillary result of this study was confirmation of the high specificity of a leptospirosis diagnosis by clinical signs and serum chemistry values at admission: *Leptospira* spp. infection was confirmed by PCR in all animals diagnosed by these methods.

Although the standard penicillin antibiotic protocol did not clear the infection, evidence suggests that alternative protocols may be effective. Elimination of leptospiruria by the time of release was positively associated with a greater duration of antibiotic administration, both calculated as the total days covered by any antibiotic and the total consecutive days covered by any antibiotic. Leptospiruria was only eliminated in California sea lions that received oxytetracycline family antibiotics (or oxytetracycline family antibiotics plus other antibiotics) in addition to the initial treatment of penicillin family antibiotics, but this effect was not statistically significant. However, sample sizes were low; thus, the power to detect significant differences was limited. In addition, these sea lions that received antibiotics from additional families also received antibiotics for a greater duration, a factor that was significantly associated with elimination of leptospiruria. Given the high degree of *in vitro* antibiotic susceptibility seen in *L. interrogans* serovar Pomona for many antibiotics used in this study (Hospenthal & Murray, 2003), investigation into the efficacy of increased duration of antibiotic administration, rather than of different antibiotics, may be the most important next step.

We have assumed that PCR positivity indicates shedding of infectious leptospires in the urine (i.e., leptospiruria). A shortcoming of our study is that we were not able to culture leptospires from any of the urine samples analyzed. Real-time PCR only detects DNA from pathogenic *Leptospira* spp., and it does not necessarily indicate viability, while culture positivity would prove that viable leptospires are present in the urine. Thus, reliance on PCR could result in overestimation of the frequency or duration of leptospiruria. We cannot

determine whether the consistent pattern of positive PCR results and negative culture results indicates a true absence of viable leptospires or if it reflects the much lower sensitivity of culture relative to PCR for *Leptospira* (Ellis, 2015). It is conceivable that genetic material continues to be shed for a period after the infection has been cleared (i.e., all leptospires are killed), but persistent shedding of *Leptospira* DNA for weeks to months after resolution of the infection seems unlikely and is not supported by the current literature (Gerritsen et al., 1993). In fact, Gerritsen et al. (1993) found that *Leptospira* infected cows treated with antibiotics became PCR-negative 2 d after treatment, while untreated cows remained PCR-positive for at least 70 d. In addition, our findings are consistent with the low sensitivity of culture from urine observed in previous studies of *Leptospira* infections in other host species. For example, of urine samples in which *Leptospira* DNA was detected via PCR, Harkin et al. (2003) had no positive cultures out of 42 PCR-positive urine samples; Prager et al. (2013) were only able to culture *Leptospira* from 6 of 32; and Bal et al. (1994) were only able to culture *Leptospira* from 5 of 12. In two of these studies (Harkin et al., 2003; Prager et al., 2013), none of the subjects had received antibiotic treatment, but culture success was still low. If antibiotic administration reduces the abundance of leptospires or inhibits their growth and, hence, decreases culture success further, our negative culture results are not surprising. Nevertheless, our data do not definitively show that sea lions were shedding viable leptospires. This is a critical issue for understanding the infectivity of animals under treatment and, thus, the potential for transmission to humans and other animals that come in contact with them; therefore, future studies should consider alternative assays for viability.

Although no culture isolates were available for identification of the infecting serovar, our MAT results strongly suggest that it was *L. interrogans* serovar Pomona. MAT results showed highest reactivity to *L. interrogans* serovar Pomona, the only serovar isolated from California sea lions to date (Zuerner & Alt, 2009), and the MAT patterns of reactivity and cross-reactivity were consistent with those detected in sea lions for decades, including individuals from which serovar Pomona was isolated (Lloyd-Smith et al., 2007).

Our findings of chronic asymptomatic carriage corroborate our earlier report of asymptomatic leptospiruria in free-ranging, wild caught California sea lions and of asymptomatic chronic leptospiruria in a stranded sea lion (Prager et al., 2013). These findings also provide further support for the hypothesis that chronic carriage is the mechanism by which *L. interrogans* serovar Pomona persists in the California sea lion population from one outbreak season to the next (Prager et al., 2013). In future studies, the addition of renal histopathology may aid in identifying changes consistent with chronic infection and carriage, and renal immunohistochemical staining could provide additional information on leptospire colonization. Finally, in assessing the role of chronic carriage in persistence, it is important to recognize that our estimates of the duration of leptospiruria are lower bounds, and the true duration may be much longer. Leptospiruria likely began prior to admission in all sea lions and persisted after release in most. In addition, PCR is an imperfect assay (< 100% sensitivity), and leptospires may be shed intermittently (Ellis, 2015); therefore, leptospiruria may not have always been detected.

Detection of chronic carriage in sea lions is also important because of their potential to transmit the pathogen. California sea lions with persistent leptospiruria represent an infection risk to others both during rehabilitation and once released back into the wild. Transmission to sea lions or to other marine mammals being rehabilitated at TMMC is possible. For example, despite precautions, four elephant seals (*Mirounga angustirostris*) being rehabilitated at TMMC developed clinical signs of leptospirosis 14 to 80 d after being admitted, suggesting that transmission occurred at TMMC (Colegrove et al., 2005). The source of infection to these elephant seals was not identified. Free-ranging wildlife may have been the source, but infected animals at TMMC are the more likely candidates since, during the same period that the four elephant seals became infected with leptospirosis at TMMC, there were two elephant seals and 25 California sea lions admitted to TMMC with presumptive diagnoses of leptospirosis (Colegrove et al., 2005; Prager, unpub. data). Seventeen of the 25 sea lions survived > 24 h, and all of these had received or were still receiving antibiotics, indicating that if they were the source of infection, they were shedding infectious leptospires despite antibiotic treatment. To date, there is no evidence of transmission to human caregivers at TMMC (Gibbins et al., 2013), although transmission of *L. interrogans* serovar Pomona from California sea lions to humans is possible (Smith et al., 1978); therefore, animal handlers at TMMC should be aware of this potential transmission risk and should take precautions to minimize this risk.

The potential transmission risk posed by animals shedding infectious leptospires underlines the importance of finding an antibiotic treatment protocol that is effective in eliminating leptospiruria and carriage. Treatment for leptospirosis in other host species frequently includes antibiotics within one of the following three families: (1) penicillin,

(2) tetracycline, and (3) cephalosporin (Watt et al., 1988; Griffith et al., 2006; Suputtamongkol et al., 2010). *In vitro* studies (Hospenthal & Murray, 2003; Murray & Hospenthal, 2004; Ressner et al., 2008; Wuthiekanun et al., 2013) have shown that many pathogenic *Leptospira* serovars, including serovar Pomona, are susceptible to antibiotics in these families. Some *in vivo* studies (McClain et al., 1984; Watt et al., 1988; Alt & Bolin, 1996; Alt et al., 2001; Truccolo et al., 2002) have shown that antibiotics within these families are effective against various pathogenic *Leptospira* serovars and may eliminate leptospiruria and possibly renal carriage. However, one of these studies also shows that treatment efficacy can differ by host species and *Leptospira* serovar. Alt & Bolin (1996) found that ceftiofur (in the cephalosporin family) and ampicillin (in the penicillin family) were not effective in clearing *L. interrogans* serovar Pomona in piglets. The same study found that in hamsters, ceftiofur, enrofloxacin (in the quinolone family), and oxytetracycline (in the tetracycline family) had to be administered at doses higher than recommended in order to be effective and that ampicillin was only effective in clearing infection in some individuals. Optimal protocols are not clearly defined for any host species, either for the treatment of symptoms of leptospirosis or for the elimination of carriage (Griffith et al., 2006; Sykes et al., 2011), and treatment protocols such as dosage and duration of treatment vary among studies, making it difficult to compare outcomes.

Our findings indicate that a 10 to 14 d course of antibiotics in the penicillin family does not eliminate leptospiruria in California sea lions infected with *L. interrogans* serovar Pomona. Bal et al. (1994) similarly detected persistent leptospiruria despite antibiotic treatment at the time of illness: urine samples from six humans that were treated with antibiotics, including two samples that were collected more than a year after the acute phase of the disease, were PCR positive for *Leptospira* DNA. In our study, treatments with longer duration and with additional antibiotics were associated with cessation of leptospiruria. It is unknown whether cessation of leptospiruria was a result of antibiotic therapy or neutralization of the pathogen by the immune system in the natural course of the disease process. A clinical trial study assessing the efficacy of different antibiotic protocols that are longer in duration or include additional or alternative antibiotics is warranted. Identification of an antibiotic protocol effective in eliminating leptospiruria in sea lions at TMMC has important human and animal health implications, and such findings would also be useful in guiding treatment of other mammalian species.

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Literature Cited

Alt, D. P., & Bolin, C. A. (1996). Preliminary evaluation of antimicrobial agents for treatment of *Leptospira interrogans* serovar Pomona infection in hamsters and swine. *American Journal of Veterinary Research*, *57*(1), 59-62.

Alt, D. P., Zuerner, R. L., & Bolin, C. A. (2001). Evaluation of antibiotics for treatment of cattle infected with *Leptospira borgpetersenii* serovar Hardjo. *Journal of the American Veterinary Medical Association*, *219*(5), 636-639. http://dx.doi.org/10.2460/javma.2001.219.636

Bal, A. E., Gravekamp, C., Hartskeerl, R. A., Demezabrewster, J., Korver, H., & Terpstra, W. J. (1994). Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. *Journal of Clinical Microbiology*, *32*(8), 1894-1898.

Colagross-Schouten, A. M., Mazet, J. A. K., Gulland, F. M. D., Miller, M. A., & Hietala, S. (2002). Diagnosis and seroprevalence of leptospirosis in California sea lions from coastal California. *The Journal of Wildlife Diseases*, *38*(1), 7-17. http://dx.doi.org/10.7589/0090-3558-38.1.7

Colegrove, K. M., Lowenstine, L. J., & Gulland, F. M. D. (2005). Leptospirosis in northern elephant seals (*Mirounga angustirostris*) stranded along the California coast. *The Journal of Wildlife Diseases*, *41*(2), 426-430. http://dx.doi.org/10.7589/0090-3558-41.2.426

Dikken, H., & Kmety, E. (1978). *Serological typing methods of leptospires*. London: Academic Press.

Ellis, W. A. (2015). Animal leptospirosis. In B. Adler (Ed.), *Current topics in microbiology and immunology:* Leptospira *and leptospirosis* (pp. 99-137). Heidelberg, Germany: Springer. http://dx.doi.org/10.1007/978-3-662-45059-8_6

Faine, S., Adler, B., Bolin, C., & Perolat, P. (1999). Leptospira *and leptospirosis* (2nd ed.). Melbourne, Australia: MediSci.

Gerber, J. A., Roletto, J., Morgan, L. E., Smith, D. M., & Gage, L. J. (1993). Findings in pinnipeds stranded along the central and northern California coast, 1984-1990. *The Journal of Wildlife Diseases*, *29*(3), 423-433. http://dx.doi.org/10.7589/0090-3558-29.3.423

Gerritsen, M. J., Koopmans, M. J., & Olyhoek, T. (1993). Effect of streptomycin treatment on the shedding of and the serologic responses to *Leptospira interrogans* serovar Hardjo subtype Hardjobovis in experimentally infected cows. *Veterinary Microbiology*, *38*(1-2), 129-138. http://dx.doi.org/10.1016/0378-1135(93)90080-Q

Gibbins, J., Niemeier, R. T., de Perio, M. A., & Mueller, C. (2013). *Health hazard evaluation report: Evaluation of zoonotic disease and exposures in persons working with marine mammals* (NIOSH HETA No. 2011-0105-33173). Cincinnati, OH: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health.

Greene, C. E., Miller, M. A., & Brown, C. A. (1998). Leptospirosis. In C. E. Greene (Ed.), *Infectious diseases of the dog and cat* (3rd ed., pp. 402-417). Philadelphia: W. B. Saunders.

Greig, D. J., Gulland, F. M. D., & Kreuder, C. (2005). A decade of live California sea lion (*Zalophus californianus*) strandings along the central California coast: Causes and trends, 1991-2000. *Aquatic Mammals*, *31*(1), 11-22. http://dx.doi.org/10.1578/AM.31.1.2005.11

Griffith, M. E., Hospenthal, D. R., & Murray, C. K. (2006). Antimicrobial therapy of leptospirosis. *Current Opinion in Infectious Diseases*, *19*(6), 533-537. http://dx.doi.org/10.1097/QCO.0b013e3280106818

Gulland, F. M. D., Koski, M., Lowenstine, L. J., Colagross, A., Morgan, L., & Spraker, T. (1996). Leptospirosis in California sea lions (*Zalophus californianus*) stranded along the central California coast, 1981-1994. *The Journal of Wildlife Diseases*, *32*(4), 572-580. http://dx.doi.org/10.7589/0090-3558-32.4.572

Harkin, K. R., Roshto, Y. M., Sullivan, J. T., Purvis, T. J., & Chengappa, M. M. (2003). Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. *Journal of the American Veterinary Medical Association*, *222*(9), 1230-1233. http://dx.doi.org/10.2460/javma.2003.222.1230

Hospenthal, D. R., & Murray, C. K. (2003). In vitro susceptibilities of seven *Leptospira* species to traditional and newer antibiotics. *Antimicrobial Agents and Chemotherapy*, *47*(8), 2646-2648. http://dx.doi.org/10.1128/AAC.47.8.2646-2648.2003

Lloyd-Smith, J. O., Greig, D. J., Hietala, S., Ghneim, G. S., Palmer, L., St. Leger, J., . . . Gulland, F. M. D. (2007). Cyclical changes in seroprevalence of leptospirosis in California sea lions: Endemic and epidemic disease in one host species? *BMC Infectious Diseases*, *7*, 125-136. http://dx.doi.org/10.1186/1471-2334-7-125

McClain, J. B., Ballou, W. R., Harrison, S. M., & Steinweg, D. L. (1984). Doxycycline therapy for leptospirosis. *Annals of Internal Medicine*, *100*(5), 696-698. http://dx.doi.org/10.7326/0003-4819-100-5-696

McIlhattan, T. J., Martin, J. W., Wagner, R. J., & Iversen, J. O. (1971). Isolation of *Leptospira pomona* from a naturally infected California sea lion, Sonoma County, California. *The Journal of Wildlife Diseases*, *7*(3), 195-197. http://dx.doi.org/10.7589/0090-3558-7.3.195

Murray, C. K., & Hospenthal, D. R. (2004). Determination of susceptibilities of 26 *Leptospira* sp. serovars to 24 antimicrobial agents by a broth microdilution technique. *Antimicrobial Agents and Chemotherapy*, *48*(10), 4002-4005. http://dx.doi.org/10.1128/AAC.48.10.4002-4005.2004

Prager, K. C., Greig, D. J., Alt, D. P., Galloway, R. L., Hornsby, R. L., Palmer, L. J., . . . Lloyd-Smith, J. O. (2013). Asymptomatic and chronic carriage of *Leptospira interrogans* serovar Pomona in California sea lions (*Zalophus californianus*). *Veterinary Microbiology*, *164*(1-2), 177-183. http://dx.doi.org/10.1016/j.vetmic.2013.01.032

R Development Core Team. (2013). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Ressner, R. A., Griffith, M. E., Beckius, M. L., Pimentel, G., Miller, R. S., Mende, K., . . . Murray, C. K. (2008). Antimicrobial susceptibilities of geographically diverse clinical human isolates of *Leptospira*. *Antimicrobial Agents and Chemotherapy*, *52*(8), 2750-2754. http://dx.doi.org/10.1128/AAC.00044-08

Roletto, J. (1993). Hematology and serum chemistry values for clinically healthy and sick pinnipeds. *Journal of Zoo and Wildlife Medicine*, *24*(2), 145-157.

Satterthwaite, F. E. (1946). An approximate distribution of estimates of variance components. *Biometrics Bulletin*, *2*(6), 110-114. http://dx.doi.org/10.2307/3002019

Smith, A. W., Vedros, N. A., Gilmartin, G., & Akers, T. G. (1978). Hazards of disease transfer from marine mammals to land mammals – Review and recent findings. *Journal of the American Veterinary Medical Association*, *173*(9), 1131-1133.

Suputtamongkol, Y., Pongtavornpinyo, W., Lubell, Y., Suttinont, C., Hoontrakul, S., Phimda, K., . . . Day, N. (2010). Strategies for diagnosis and treatment of suspected leptospirosis: A cost-benefit analysis. *PLOS Neglected Tropical Diseases*, *4*(2), e610. http://dx.doi.org/10.1371/journal.pntd.0000610

Sykes, J. E., Hartmann, K., Lunn, K. F., Moore, G. E., Stoddard, R. A., & Goldstein, R. E. (2011). 2010 ACVIM small animal consensus statement on leptospirosis: Diagnosis, epidemiology, treatment, and prevention. *Journal of Veterinary Internal Medicine*, *25*(1), 1-13. http://dx.doi.org/10.1111/j.1939-1676.2010.0654.x

Truccolo, J., Charavay, F., Merien, F., & Perolat, P. (2002). Quantitative PCR assay to evaluate ampicillin, ofloxacin, and doxycycline for treatment of experimental leptospirosis. *Antimicrobial Agents and Chemotherapy*, *46*(3), 848-853. http://dx.doi.org/10.1128/AAC.46.3.848-853.2002

Vedros, N. A., Smith, A. W., Schonewald, J., Migaki, G., & Hubbard, R. C. (1971). Leptospirosis epizootic among California sea lions. *Science*, *172*(3989), 1250-1251. http://dx.doi.org/10.1126/science.172.3989.1250

Watt, G., Padre, L. P., Tuazon, M. L., Calubaquib, C., Santiago, E., Ranoa, C. P., & Laughlin, L. W. (1988). Placebo-controlled trial of intravenous penicillin for

severe and late leptospirosis. *Lancet*, *1*(8583), 433-435. http://dx.doi.org/10.1016/S0140-6736(88)91230-5

Welch, B. L. (1947). The generalization of "Student's" problem when several different population variances are involved. *Biometrika*, *34*(1-2), 28-35. http://dx.doi.org/10.1093/biomet/34.1-2.28

Wu, Q., Prager, K. C., Goldstein, T., Alt, D. P., Galloway, R. L., Zuerner, R. L., . . . Schwacke, L. (2014). Development of a real-time PCR for the detection of pathogenic *Leptospira* spp. in California sea lions. *Diseases of Aquatic Organisms*, *110*(3), 165-172. http://dx.doi.org/10.3354/dao02752

Wuthiekanun, V., Amornchai, P., Paris, D. H., Langla, S., Thaipadunpanit, J., Chierakul, W., . . . Peacock, S. J. (2013). Rapid isolation and susceptibility testing of *Leptospira* spp. using a new solid medium, LVW agar. *Antimicrobial Agents and Chemotherapy*, *57*(1), 297-302. http://dx.doi.org/10.1128/AAC.01812-12

Zuerner, R. L., & Alt, D. P. (2009). Variable Nucleotide Tandem-Repeat analysis revealing a unique group of *Leptospira interrogans* serovar Pomona isolates associated with California sea lions. *Journal of Clinical Microbiology*, *47*(4), 1202-1205. http://dx.doi.org/10.1128/JCM.01639-08