

DIAGNOSIS AND SEROPREVALENCE OF LEPTOSPIROSIS IN CALIFORNIA SEA LIONS FROM COASTAL CALIFORNIA

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ABSTRACT: The sensitivity and specificity of the microscopic agglutination test (MAT) as a method for detection of exposure to *Leptospira* spp. in California sea lions (*Zalophus californianus*) were determined. Sera came from individuals that demonstrated clinical signs of renal disease, had lesions suggestive of leptospirosis at necropsy, and had visible leptospire in silver stained kidney sections as positive controls. Sera from unexposed captive individuals were used as negative controls. The test was 100% sensitive at 1:3,200 for confirming renal infection and 100% specific at negative <1:100 for detection of *Leptospira interrogans* serovar pomona antibodies by MAT in California sea lions. *Leptospira interrogans* serovar pomona was used as a screening serovar because it has been isolated previously from the kidneys and placentas of California sea lions, and there appears to be cross-reactivity between serovar pomona and other serovars. Sera from 225 free-ranging California sea lions presented to one of three participating California (USA) coastal marine mammal rehabilitation centers in 1996 were then evaluated for antibodies to serovar pomona using the MAT. The overall seroprevalence was 38.2% (86/225), although the prevalence varied among locations from 100% (38/38) in animals at the Marine Mammal Care Center (Fort MacArthur, California, USA) to 0% (0/14) at SeaWorld California (San Diego, California). At The Marine Mammal Center (Sausalito, California) [prevalence 27.8% (48/173)], the majority of seropositive animals were subadults and adults, and males were 4.7 times more likely to be seropositive to serovar pomona than females. When combining results from all three centers, subadult and adult animals were more likely to be seropositive than pups and juvenile sea lions, and the highest proportion of seropositive animals presented during the autumn months. Serum elevations of blood urea nitrogen, creatinine, phosphorus, and/or calcium were associated with seropositivity to serovar pomona. We found no association between potassium or sodium levels and seropositivity.

Key words: California sea lion, leptospirosis, *Leptospira interrogans* pomona, microscopic agglutination test, seroprevalence, Warthin-Starry stain, *Zalophus californianus*.

INTRODUCTION

Leptospirosis is a zoonotic disease that infects a great number of both domestic and wild animal species. Susceptible wildlife include fieldmice, hedgehogs, foxes, frogs, wild boars, vervet monkeys (*Cercopithecus aethiops sabaues*), white-tailed deer (*Odocoileus virginianus*) and marine mammals, such as Northern fur seals (*Callorhinus ursinus*) (Smith et al., 1977; Baulu et al., 1987; Andre-Fontaine and Ganire, 1990; New et al., 1993). Serovars of *Leptospira* spp. may be categorized as host adapted and non-host adapted (Heath and Johnson, 1994). Infection with host adapted serovars of *Leptospira* spp. usually results in the development of mild, subclin-

ical disease, and abortion. The infected animal may continue to shed the organism for its entire lifetime. It may serve as a source of infection to naive members of the same species, creating more maintenance hosts. In contrast, infection with non-host adapted serovars usually results in sporadic infection with acute signs of severe disease and a low prevalence of seropositive hosts that shed leptospire in the urine for a shorter time period (Heath and Johnson, 1994).

Several severe outbreaks of renal disease resulting in hundreds of stranded California sea lions along the coast of California (USA) have been attributed to *Leptospira interrogans* serovar pomona, the

most commonly isolated serovar from California sea lions (Medway, 1980). High numbers of apparently affected individuals were observed in 1970, 1984, 1988, 1991, and 1994 (Vedros et al., 1971; Dierauf et al., 1985; Gerber et al., 1993; Gulland et al., 1996). One of the most commonly used methods to establish a diagnosis of leptospirosis in domestic animals is the microscopic agglutination test (MAT) on sera. However, only sera from a small percentage of the animals diagnosed with renal disease based on clinical signs (severe depression, emaciation, extreme thirst, and tucked-up abdomens) upon presentation to marine mammal rehabilitation centers were tested with the MAT. When a small number of selected individuals tested positive on the MAT, all animals presenting with signs of renal disease during that epidemic period were presumed to be infected with serovar pomona (Dierauf et al., 1985; Gulland et al., 1996). This extrapolation to all similarly presenting animals may have been problematic as other causes of renal disease occur in sea lions, the background rate of *Leptospira* spp. exposure was not determined, and no control animals were tested. Furthermore, the MAT was originally developed for cattle. Therefore, its sensitivity and specificity for use in detection of *Leptospira* spp. exposure in California sea lions had not been established. The validation of this diagnostic tool for the characterization of outbreaks of disease presumed to be caused by serovar pomona was thus warranted.

Epidemics of leptospirosis in California sea lions have been considered to be a regional problem, thought to be restricted to the northern coast of California (Sweeny and Gilmartin, 1974; Howard et al., 1983; Gulland et al., 1996). Whether this regional predilection was due to the actual absence of the disease in other portions of the range, the seasonality of clinical expression of disease when animals are concentrated in certain regions, or differences in clinical approach and management of stranded sea lions among the different re-

habilitation centers in California is unknown. Information about the prevalence of *Leptospira* spp. exposure in California sea lions throughout the California coast may help us to further understand the epidemiology of this disease.

Our objectives in this study were to assess the validity of the *L. interrogans* serovar pomona MAT for detecting exposure to leptospires in California sea lions stranded along the California coast, and to evaluate the prevalence of exposure in sea lions presenting to California marine mammal rehabilitation centers in 1996. We also investigated serum chemistry values as indicators of renal disease associated with leptospire exposure.

MATERIALS AND METHODS

Assessment of the MAT

To evaluate the sensitivity of the MAT for detection of exposure to *Leptospira* spp. in California sea lions, kidney samples were obtained from 40 California sea lions that died at The Marine Mammal Center (TMMC; Golden Gate National Recreation Area, Sausalito, California, USA) from 1994–96. The sea lions had clinical signs consistent with renal disease, were found to have lesions suggestive of leptospirosis on subsequent necropsy, and had serum banked. Kidney samples were fixed by immersion in 10% neutral buffered formalin and later embedded in TissuePrep (Fisher Scientific, Fairlawn, New Jersey, USA) and sectioned at 6 μ m. Two slides were prepared from each of the 40 kidney samples. Slides were stained according to the methods described by Kerr (1938) with the following modifications. Warthin Starry silver staining was achieved by placing the slides in a plastic Coplin jar with 1% silver nitrate and heating them in a H2200 Staining Microwave (Energy Beam Sciences, Inc., Agawam, Massachusetts, USA) for 35 sec at 32 C. Slides were then placed in another Coplin jar with developer solution prepared with 10 ml of 2% silver nitrate, 24.8 ml of 5% gelatin, and 13.2 ml of 0.2% hydroquinone and heated in the H2200 Staining Microwave for 25 sec at 32 C. Slides were microscopically examined at 100 \times for the presence of darkly stained, slender or filamentous spiral shaped organisms compatible with spirochetes. Nineteen of the 40 California sea lions were identified as positive controls based on their selection as a case and on microscopic identification of leptospires in kidney tissues stained by the Warthin-Starry

staining technique. For further confirmation of the staining technique six randomly-selected, paraffin-embedded blocks of kidney were submitted in a blind manner to the Georgia Veterinary Diagnostic Laboratory (GVDL; Athens, Georgia) for *Leptospira*-specific immunoperoxidase staining. Stains were performed according to standard laboratory techniques (Tizard, 1987). Briefly, 5 μ m thick sections were cut on a rotary microtome and placed on glass slides. The sections were deparaffinized, then incubated with rabbit polyclonal antiserum to serovar pomona (National Veterinary Services Laboratory, Ames, Iowa) for 30 min at a 1:50,000 dilution. After a phosphate-buffered saline (PBS) wash, the slides were incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, California; 1:100 dilution) for 25 min. Washed slides were coated with extra-avidin (Sigma, St. Louis, Missouri, USA) for 15 min, followed by the chromagen, 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 12 min. An automated immunostainer (BioTek 55, BioTek Solutions Inc., Santa Barbara, California) was used to complete the avidin-biotin staining protocol (Tizard, 1987).

In order to establish MAT specificity, negative control individuals were selected. These included captive bred animals that had never been in contact with free-ranging animals and that had never demonstrated clinical signs of renal disease. Also, we included captive individuals that had never demonstrated clinical signs of renal disease, and that had not been in contact with free-ranging individuals for >10 yr. Four serum samples from negative control California sea lions were obtained from Oceanic Park (Aberdeen, Hong Kong), 10 from SeaWorld Ohio (Aurora, Ohio, USA), and five from the Brookfield Zoo (Brookfield, Illinois, USA). The 19 negative control and 19 positive control samples were submitted to the California Animal Health and Food Safety Laboratory (CAHFS; University of California, Davis, California) in a blind manner for MAT. Sensitivity, specificity, and positive and negative predictive values for the MAT were calculated according to methods described by Smith (1995).

1996 regional survey

Stranded California sea lions presented to participating marine mammal rehabilitation centers along the California coast between 1 January 1996 and 31 December 1996 were included in this study if they were examined clinically, had a blood sample drawn, and had serum stored. Animals were presented to one of three rehabilitation centers, depending upon

their stranding location. Individual animals stranding along the coast from 37°42'N, 123°05'W to 35°59'N, 121°30'W were presented to TMMC. The Marine Mammal Care Center at Fort MacArthur (MMCC; San Pedro, California) collected animals along the coast from 34°17'N, 119°30'W to 33°45'N, 118°07'W. SeaWorld California (SW; San Diego, California) collected animals along the coast from 33°45'N, 118°07'W to 32°32'N, 117°07'W. Age category and sex of each individual were determined by examination of body length, weight, sagittal crest development, pelage coloration, and external genital morphology (Mate, 1978). Animals were classified <1-yr-old as pups, 1- to 2-yr-old as juveniles, 3- to 5-yr-old as subadults, and >5 yr as adults.

Blood samples were obtained in most cases from the caudal gluteal vein using an 18 gauge \times 38 mm needle (Bossart and Dierauf, 1990). If the animal was presented to the rehabilitation center dead or was found dead during the rehabilitation period, an attempt to obtain heart blood was made using a 18 gauge \times 75 mm needle inserted into a right intercostal space from ribs 3 to 5 (Bossart and Dierauf, 1990). Blood samples were typically collected upon admission. Samples were not collected on every California sea lion presented in 1996, as some animals were dead upon arrival to the centers, appropriate blood collection personnel were not always available, and some fractious animals could not be handled. Furthermore, a sufficient amount of serum had to be available for MAT after other clinical pathological procedures were performed because the majority of animals were bled only one time. At TMMC, 173 of 214 California sea lions had a blood sample taken and serum stored. At the MMCC, 38 of 64 animals had serum stored, and at SW 14 of 31 had a serum sample stored in 1996.

Blood samples were placed into vacutainers containing serum separation gel and clot activator (Vacutainer, Becton Dickinson, Rutherford, New Jersey, USA) and were centrifuged at 3,000 \times G within 2 hr of collection to separate serum. Serum samples were removed from the collection vacutainers, placed into bio-freeze vials (Costar Corporation, Cambridge, Massachusetts, USA) and frozen at -20 C for future analysis. Serum biochemistry analyses were performed on a Vet test 8008 (Idexx Laboratories Inc., Westbrook, Maine, USA) or a 747-100 (Hitachi, Boehringer Mannheim Laboratory Diagnostics, Indianapolis, Indiana, USA). Serum electrolytes were measured on a VetLyte Electronic Analyser (Idexx Laboratories Inc.) or a 747-100 (Hitachi).

Stored serum was submitted to CAHFS for MAT using serovar pomona antigen as a

screening tool for California sea lion leptospirosis at a dilution of 1:100 (Galton et al., 1962). Animals that had demonstrable titers $\geq 1:100$ were considered to be exposed. Information including stranding date and location, disposition, and serum biochemistry data, when available, was recorded for individuals included in the study.

An overall prevalence of exposure among the animals included in this study was calculated, as well as the prevalence for each center. Chi-square analysis was used to measure the strength of association between seropositivity and the factors of age (<1 yr, 1 to 2 yr, 3 to 5 yr, and >5 yr), and stranding season (winter, spring, summer, and fall). Odds ratios (OR) and 95% confidence intervals (CI) were used to measure the strength of association between seropositivity and sex (males and females). Analysis was performed using EpiInfo statistical software (Version 6.02–October 1994, Center for Disease Control and Prevention, Atlanta, Georgia) and values were established for each center and for all three centers combined.

If biochemistry panels were performed, values for blood urea nitrogen (BUN), creatinine, phosphorus, potassium, sodium, and calcium were recorded as these values are used to clinically assess renal function. Biochemistry values for each individual were classified as normal if there were concentrations of BUN ≤ 50 mg/dl, creatinine ≤ 1 mg/dl, phosphorus ≤ 7 mg/dl, calcium ≤ 8.9 mg/dl, potassium ≤ 4.2 mEq/l, and sodium ≤ 151 mEq/l (Roletto, 1993; Gulland et al., 1996). Odds ratios for each measured biochemistry value were calculated in order to establish the strength of association between exposure status and clinical measurements of renal disease (Fleiss, 1981).

RESULTS

Assessment of the MAT

All 19 animals used as positive controls due to visualization of leptospires in silver stained sections of kidney had a serum titer to *L. pomona* of $>1:3,200$ as detected by MAT. All negative control serum samples from California sea lions in captivity were negative ($n = 19$) for exposure to *L. pomona* by MAT. Therefore, the positive and negative predictive value of the MAT in identifying an individual with a titer of $\geq 1:3,200$ to *L. pomona* are both equal to one, and the sensitivity and specificity of the MAT were both 100% for this study.

As a confirmation of the specificity of

the Warthin Starry staining technique, immunoperoxidase staining for *Leptospira* spp. antibody using polyclonal antibody originally developed against *L. pomona* antigen was performed by the GVDL on sections of kidney from four of the confirmed positive animals. Positive staining was noted on all four slides. One slide from a seronegative animal that did not have leptospires visualized with silver staining was also submitted, and no immunoperoxidase staining was noted. A slide from an individual with a *L. pomona* titer of $>1:3,200$ and no leptospires visualized with the Warthin Starry stain was also submitted for immunoperoxidase stain and revealed strong positive staining.

The Warthin-Starry stain allowed for visualization of the leptospires within renal tubules and immunoperoxidase staining (GVDL) confirmed the presence of *Leptospira* spp. antigen in the kidney. Secondary findings such as inflammation of associated renal tubules were noted (Fig. 1).

1996 Regional Survey

The overall prevalence of exposure in stranded California sea lions seen at TMMC with a titer to serovar *pomona* $\geq 1:100$ was 27.8% (48/173; Table 1). A significant association ($\chi^2 = 36.1$; 3 df, $P < 0.01$) between seropositivity and age was found, with 66.7% (16/24) of stranded subadults and 57.9% (11/19) of adults found seropositive compared to 0% (0/6) of pups and 16.9% (21/124) of juveniles. There was a significant strength of association between seropositivity and sex, with males being 4.7 times more likely to be seropositive to *L. pomona* exposure than females (OR = 4.7; 95% CI = 1.7 to 13.2). There also was a significant association between seropositivity and season of stranding ($\chi^2 = 68.0$; 3 df, $P < 0.01$), with 91.3% (21/23) of those presenting in autumn months and 100% (3/3) of those animals presenting in winter months being seropositive for serovar *pomona* exposure.

The overall prevalence in stranded California sea lions seen at MMCC with a ti-

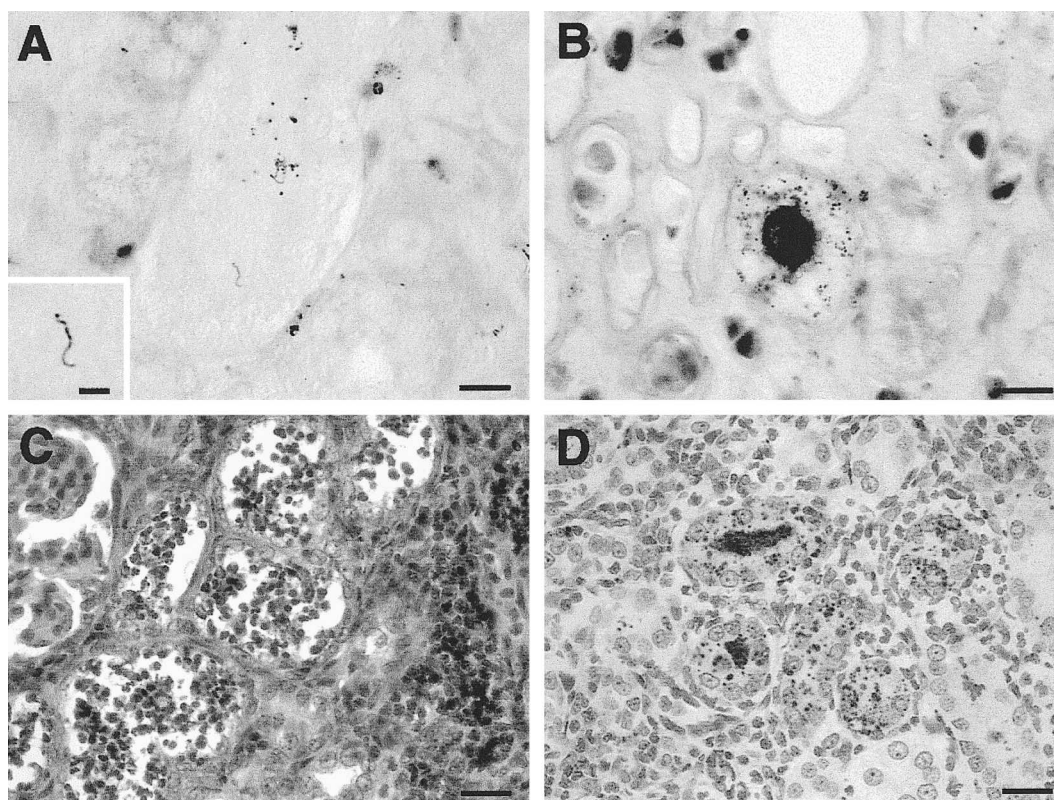


FIGURE 1. Photomicrographs of the renal cortex taken from a kidney section from a California sea lion with a titer to *Leptospira interrogans* serovar pomona of $>1:3,200$. A. Warthin-Starry stain reveals individual leptospires within a renal tubule. Bar = 40 μm . Inset detail of single leptospire shows classic morphology, including terminal hook. Bar = 40 μm . B. A dense aggregate of leptospires is visible with Warthin-Starry staining in a cross-section of a renal tubule. Bar = 40 μm . C. H&E stained section reveals prominent tubular ectasia, lymphoplasmacytic and neutrophilic tubulointerstitial nephritis and tubular epithelial necrosis with early evidence of epithelial regeneration. Bar = 20 μm . D. A section stained with an immunoperoxidase stain that uses antibodies directed against serovar pomona. There is prominent intratubular and intraepithelial staining in the interstitial inflammatory cells. This globular staining pattern was typical of all known positive sea lion cases stained by this method. Bar = 20 μm .

ter to serovar pomona $\geq 1:100$ was 100% (38/38; Table 1). Of these animals, 63.2% (24/38) were juveniles with 87.5% (21/24) of juveniles being female. Of all seropositive animals, 73.7% (28/38) were female. The majority of these animals presented during the spring and summer months (33/38).

None of the stranded California sea lions received at SW had a detectable titer to *L. pomona* (0/14; Table 1). Of these stranded animals, 42.9% (6/14) were juveniles and 35.7% (5/14) were adults. Slightly more males (57.1%; 8/14) were presented to SW. Five of the 14 (35.7%)

animals were presented in the winter months with equal numbers (3/14; 21.4%) presented in spring, summer, and fall.

The overall prevalence in stranded California sea lions seen at participating marine mammal rehabilitation centers in 1996 with a titer to serovar pomona $\geq 1:100$ was 38.2% (86/225; Table 1). A significant association ($\chi^2 = 19.49$, 3 df, $P < 0.01$) between seropositivity and age was found with 64.5% (19/29) of stranded subadults and 58.1% (18/31) of adults seropositive compared to 36.4% (4/11) pups and 29.2% (45/154) juveniles having titers to *L. pomona*. There was a significant

TABLE 1. Percent (number exposed/number tested) of California sea lions seropositive to *Leptospira interrogans* serovar pomona by marine mammal rehabilitation center (MMRC), sex, age, and stranding season in 1996.

| | MMRC | Sex | | | Age | | | | Stranding season | | | |
|-------------------|-------------------|-------------------|------------------|-----------------|-------------------|------------------|------------------|----------------|-------------------|------------------|------------------|--|
| | | Male | Female | Pup | Juvenile | Subadult | Adult | Winter | Spring | Summer | Autumn | |
| TMMC ^a | 27.8% (48/173) | 35.9% (42/117) | 10.7% (6/56) | 0% (0/6) | 16.9% (21/124) | 66.7% (16/24) | 57.9% (11/19) | 100% (3/3) | 9.6% (8/83) | 25.0% (16/64) | 91.3% (21/23) | |
| MMCC ^b | 100% (38/38) | 100% (10/10) | 100% (28/28) | 100% (4/4) | 100% (24/24) | 100% (3/3) | 100% (7/7) | 100% (1/1) | 100% (23/23) | 100% (10/10) | 100% (4/4) | |
| SW ^c | 0% (0/14) | 0% (0/8) | 0% (0/6) | 0% (0/1) | 0% (0/6) | 0% (0/2) | 0% (0/5) | 0% (0/3) | 0% (0/3) | 0% (0/3) | 0% (0/3) | |
| Total | 38.2% (86/225) | 38.5% (52/135) | 37.8% (34/90) | 36.4% (4/11) | 29.2% (45/154) | 65.5% (19/29) | 58.1% (18/31) | 44.4% (4/9) | 28.4% (31/109) | 33.8% (26/77) | 83.3% (25/30) | |

^a The Marine Mammal Center.
^b Marine Mammal Care Center.
^c Sea World California.

strength of association between seropositivity and season of the year ($\chi^2 = 38.79$, 3 df, $P < 0.01$), with 83.3% (25/30) of those animals presented in autumn months being seropositive for serovar pomona, as compared to 44.4% (4/9), 28.4% (31/109), and 33.8% (26/77) in the winter, spring, and summer, respectively. No significant strength of association was found between seropositivity and sex ($P > 0.5$).

Serum chemistry values

Serum chemistries were submitted from 113 of 225 California sea lions presented to marine mammal rehabilitation centers in 1996 (Table 2). California sea lions with a BUN value >50 mg/dl were 15.9 times more likely to be seropositive to serovar pomona than those with a BUN ≤ to 50 mg/dl (OR = 15.9; 95% CI = 4.6 to 58.7). California sea lions with a creatinine value >1 mg/dl were 25.8 times more likely to be seropositive to serovar pomona than those with a creatinine of ≤1 mg/dl (OR = 25.8; 95% CI = 6.8 to 107.0). California sea lions with a phosphorus value >7 mg/dl were 6.8 times more likely to be seropositive to serovar pomona than those with a phosphorus of less than or equal to 7 mg/dl (OR = 6.8; 95% CI = 2.4 to 20.3); sea lions with a calcium value >8.9 mg/dl were 16.8 times more likely to be seropositive to serovar pomona than those with a lower calcium concentration (OR = 16.8; 95% CI = 4.3 to 70.0). No statistically significant associations were found between seropositivity and the levels of potassium (OR = 2.3; 95% CI = 0.8 to 6.1) or sodium (OR = 1.7; 95% CI = 0.7 to 4.3) using the cutoff values of greater than 4.2 mEq/l and 151 mEq/l, respectively.

DISCUSSION

Although the MAT was developed for use in cattle, it has been used in California sea lions for many years without knowledge about the test's sensitivity and specificity in this species. Our results confirm that the MAT is a useful and accurate diagnostic tool for detection of serovar po-

TABLE 2. Percent (number exposed/number tested) of California sea lions with elevated serum chemistry and electrolyte values by exposure groups (seropositive or seronegative) to *Leptospira interrogans* serovar *pomona* by the microscopic agglutination test in 1996.

| | BUN >50 mg/dl | Creatinine >1 mg/dl | Phosphorus >7 mg/dl | Calcium >8.9 mg/dl | Potassium > 4.2 mEq/l | Sodium >151 mEq/l |
|--------------|-------------------|------------------------|------------------------|-----------------------|--------------------------|----------------------|
| Seropositive | 51.5% (17/33) | 57.6% (19/33) | 76.7% (23/30) | 60.0% (12/20) | 40.0% (12/30) | 53.3% (16/30) |
| Seronegative | 6.3% (5/80) | 5.0% (4/80) | 32.5% (26/80) | 8.2% (6/73) | 22.8% (18/79) | 40.5% (32/79) |
| Total | 19.5% (22/113) | 20.4% (23/113) | 44.6% (49/110) | 19.4% (18/93) | 27.5% (30/109) | 44.0% (48/109) |

mona exposure in California sea lions. Serovar *pomona* was chosen to screen all serum samples by MAT, as it was the organism previously isolated from the kidneys of California sea lions presenting with clinical signs of renal disease (McIlhattan et al., 1971; Gage et al., 1993; Gulland et al., 1996) and from the kidneys and placentas of aborted sea lion fetuses (Smith et al., 1974; Gilmartin et al., 1976). Previous diagnostic efforts from 1991–93 have included analysis of samples for exposure to multiple serovars including grippotyphosa, bratislava, and icterohaemorrhagiae. To our knowledge, in almost every case, samples positive to one or more of these serovars also had high titers ($\geq 1:3,200$) to serovar *pomona* (Gerber et al., 1993; Gulland et al., 1996). Therefore, it is reasonable to assume that there is a degree of antibody cross-reactivity among serotypes, as is documented in other host species (Swart et al., 1982; Brown et al., 1996). Although it is impossible to rule out that the MAT is detecting a novel marine leptospire that is highly cross-reactive with serovar *pomona*, we have no evidence to suggest that such an organism exists. Therefore, testing for exposure to serovar *pomona* should serve as a valuable screening tool. Screening large numbers of animals for only one serovar is more economical in a rehabilitation setting and, given cross-reactivity, provides enough information for instituting therapy.

Our finding of 100% sensitivity at a titer of 1:3,200 to serovar *pomona* by MAT suggests that the test is useful and accurate (n

= 19 exposed) in detecting infection based on our positive control of renal disease with demonstrated leptospire in kidney sections. A 100% specificity was predicted at the negative 1:100 cut-off based on the negative control population of non-diseased, unexposed California sea lions ($n = 19$). Our standard population did not allow us to assign interpretation or sensitivity and specificity values between 1:100 and 1:1,600. However, the majority of individuals in this study with a titer to serovar *pomona* had high titers $> 1:3,200$ (73.3%; 63/86), consistent with our positive control population. Because no unexposed captive animals in our negative control population had detectable titers ($\geq 1:100$), we decided to classify all individuals with titers $\geq 1:100$ as exposed for screening purposes.

In the past, epidemics of leptospirosis in California sea lions have been considered a regional problem, restricted to the northern coast of California, with more males presented than females (Dierauf, 1983; Gerber et al., 1993; Gulland et al., 1996). Although only three of the six California marine mammal rehabilitation centers participated in this study, we found that exposure was widely distributed along the coast of California. The overall prevalence of exposure for these California rehabilitation centers in 1996 was 38.2%. This prevalence was most closely approximated by TMMC at 27.8%, with the prevalence at MMCC being 100%. At SW, none of the animals tested had a titer to serovar *pomona* in 1996. This result was unexpected

because of the high prevalence at the other southern California marine mammal center (MMCC), so sera from past years collected at SW were evaluated to determine whether this result might have been due to the small sample size, to annual or seasonal variation of the sea lions presented, or to the absence of serovar pomona. Of 39 samples submitted from 1991, 1993, 1994, and 1995, 21 were positive with MAT (20/21 had titers $>1:3,200$). Individuals presented to SW were distributed across sexes, age classes, and seasons. Therefore, we believe our 1996 finding of no detectable response to serovar pomona is likely due to our small regional sample size ($n = 14$) rather than a true absence of exposure in San Diego in 1996.

Previous studies have demonstrated that California sea lion males at TMMC are exposed in higher numbers to *Leptospira* spp. than females (Dierauf et al., 1985; Gulland et al., 1996). In our study, we found these previous findings at TMMC to hold true, with males being nearly five times more likely to have titers to serovar pomona than females. This result may be confounded, however, by the fact that more males were presented to TMMC (67.6%) than to the MMCC (26.3%) or to SW (57.1%). More subadults and adults were seropositive ($P < 0.01$), even though the majority were juveniles. There was a significant association between seropositivity and season ($P < 0.01$), with the highest proportions of positive animals presented in autumn and winter even though the majority of animals were presented in the spring and summer. It should be noted, however, that our sample size for the winter months was small ($n = 3$) as a result of the lower 1996 caseload at TMMC during the winter. In future years it would be helpful to explore the apparent increased prevalence of exposure in winter.

Although it was previously believed that more male California sea lions were exposed to serovar pomona, it appears that association by sex may vary by location or

another unknown factor. The only statistically significant finding was at TMMC where more males strand for rehabilitation. There was no significant difference between seropositivity and sex overall. Perhaps more males than females are found stranded in northern California because they are more migratory. Therefore, they may travel longer distances from the islands off the southern California coast where breeding takes place (Riedman, 1990). Similarly, wild-caught male vervet monkeys were found to be seropositive to *Leptospira* spp. in significantly higher numbers than females. This finding may be attributable to male behavior, as males migrate to other groups and are known to travel longer distances than females while foraging (Baulu et al., 1987).

Although the age class distribution was similar among all centers, with the majority of animals in the juvenile class, there were significantly more subadults and adults exposed to serovar pomona. Differences in the rate of exposure among age classes may be due to the fact that adults and subadults have had a longer time period in which to become exposed to serovar pomona. This scenario would be true if this serovar was host adapted, as in horses, where the risk of exposure increases with age (Lees and Gale, 1994). Similarly, once infected, cattle may retain renal carrier status for months to years (Thiermann, 1982) and would be seropositive during this time. However, the chronic carrier state in California sea lions seems unlikely because of the high titers ($>1:3,200$) found in the majority of seropositive animals (73.3%; 63/86). These high titers are indicative of recent exposure, as chronically infected animals with a host-adapted serovar in other species studied tend to have low titers (Heath and Johnson, 1994). Sea lions likely first encounter the organism within an adult colony and are continuously re-exposed due to thigmotactic behavior, laying in close approximation and being consistently exposed to urine from conspecifics (Peterson and Barthol-

omew, 1967). Therefore, adults become reinfected every year. Further evidence for reexposure from conspecifics is suggested by Baulu (1987). He found that when monitoring an individual wild-caught vervet monkey over several years, the titer peaked during captivity in a group-housing situation.

The majority of mid-range titers (1:100–1:1,600) were detected in males that stranded along the northern California coast (82.6%; 19/23). This finding may be due to the fact that these males have traveled longer distances and may have stranded a longer time period after exposure than conspecifics stranding in southern California.

Increases in seropositivity in autumn and winter months concur with the seasonal pattern of leptospirosis observed in stranded California sea lions in central California (Gulland et al., 1996), although the majority of strandings there were in spring and summer. Increases in seropositivity in autumn and winter may be explained by increased energy demands following reproduction, migratory behavior or changes in the food supply resulting in immunocompromise, making the animals more susceptible to exposure and infection during this time. Sea lions are on the rookeries during the late spring and summer months which brings animals in closer approximation with conspecifics for breeding, permitting exposure to individuals shedding leptospire. When sea lions leave the rookeries in late summer and move northward, they also achieve close contact on haul-out areas. The reasons for a higher proportion of seropositive animals presented in the autumn and winter are unclear. The possibility of direct transmission exists on the rookeries in summer as well as the haul-out areas in the autumn.

The relationship between season and exposure has also been examined in cattle (Miller et al., 1991), and although the majority of isolates were obtained during summer and autumn months, there were no statistically significant differences among

seasons. Studies on humans have found that individual factors may strongly influence the progress of the agglutinating antibody response (Lupidi et al., 1991). We feel that the predictability of the host adapted versus non-host adapted serovar model is not definitive for California sea lions and probably subject to many variables. It is likely that there are multiple factors influencing exposure of California sea lions and further study on the duration of detectable antibody titers and leptospiuria is warranted.

Serum chemistries are useful in determining whether an animal demonstrating clinical signs consistent with leptospirosis may actually be suffering from renal disease. Rehabilitation centers can use chemistry results to determine the severity of renal disease and direct appropriate treatment. The serum chemistry results in this study indicate that elevations in BUN, creatinine, phosphorus, and calcium were significantly associated with exposure to serovar pomona, especially serum creatinine. Therefore, it is reasonable to presume that elevations in these kidney parameters in association with a high titer to serovar pomona would suggest that an animal has renal disease resulting from leptospirosis.

In summary, we found that the MAT is a useful tool for detecting *Leptospira* spp. antibodies in California sea lions and that lack of a specific MAT response is effective in ruling out infections with *Leptospira* spp. It is clear that California sea lions exposed to *Leptospira* spp. strand in many locations along the California coast, and that in 1996, adults and subadults were more likely to be seropositive than juveniles and pups. Further serosurveys are needed to clarify temporal and spatial changes in seroprevalence, which in turn may help to elucidate the epidemiology of leptospirosis in California sea lions. Although serovar pomona is the organism that has been cultured from infected California sea lions, we cannot rule out the possibility that the MAT is detecting a novel marine leptospire that is highly cross-

reactive with serovar pomona. Clinical chemistries are helpful for detecting renal disease in animals presented with clinical signs of leptospirosis, to expedite treatment, and to take handling precautions to minimize risk of disease spread while awaiting results from MAT.

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LITERATURE CITED

- ANDRE-FONTAINE, G., AND J. P. GANIERE. 1990. New topics on leptospirosis. *Comparative Immunology, Microbiology and Infectious Diseases* 13: 163–168.
- BAULU, J., C. O. R. EVERARD, AND J. D. EVERARD. 1987. Leptospire in vervet monkeys (*Cercopithecus aethiops sabaesus*) on Barbados. *Journal of Wildlife Diseases* 23: 60–66.
- BOSSART, G. D., AND L. A. DIERAUF. 1990. Marine mammal clinical laboratory medicine. In *Handbook of marine mammal medicine: Health, disease, and rehabilitation*, L. A. Dierauf (ed.). CRC Press Inc., Boca Raton, Florida, pp. 1–52.
- BROWN, C. A., A. W. ROBERTS, M. A. MILLER, D. A. DAVIS, S. A. BROWN, C. A. BOLIN, J. JARECKI-BLACK, C. E. GREENE, AND D. MILLER-LIEBL. 1996. *Leptospira interrogans* serovar *grippotyphosa* infection in dogs. *Journal of the American Veterinary Medical Association* 209: 1265–1267.
- DIERAUF, L. A. 1983. A survey of live pinnipeds stranded along the northern California coast. *California Veterinarian* 6: 22–26.
- , D. J. VADENBROEK, J. ROLETTO, M. KOSKI, L. AMAYA, AND L. J. GAGE. 1985. An epizootic of leptospirosis in California sea lions. *Journal of the American Veterinary Medical Association* 187: 1145–1148.
- FLEISS, J. L. 1981. *Statistical methods for rates and proportions*, 2nd Edition. John Wiley and Sons, New York, New York, 321 pp.
- GAGE, L. J., J. A. GERBER, D. M. SMITH, AND L. E. MORGAN. 1993. Rehabilitation and treatment success rate of California sea lions (*Zalophus californianus*) and northern fur seals (*Callorhinus ursinus*) stranded along the central and northern California coast, 1984–1990. *Journal of Zoo and Wildlife Medicine* 24: 41–47.
- GALTON, M. M., R. W. MENGES, E. B. SCHOTTS, A. J. NATMIAS, AND C. W. HEATH. 1962. *Leptospirosis: Epidemiology, clinical manifestations in man and animals, and methods in laboratory diagnostics*. U.S. Public Health Service Publication No. 951, Washington, D.C., 70 pp.
- GERBER, J. A., J. ROLETTO, L. E. MORGAN, D. M. SMITH, AND L. E. MORGAN. 1993. Findings in pinnipeds stranded along the central and northern California coast, 1984–1990. *Journal of Wildlife Diseases* 29: 423–433.
- GILMARTIN, W. G., R. L. DELONG, A. W. SMITH, J. C. SWEENEY, B. W. DE LAPPE, R. W. RISEBROUGH, L. A. GRINER, M. D. DALEY, AND D. B. PEAKALL. 1976. Premature parturition in the California sea lion. *Journal of Wildlife Diseases* 12: 104–115.
- GULLAND, F. M. D., M. KOSKI, L. J. LOWENSTINE, A. COLAGROSS, L. MORGAN, AND T. SPRAKER. Leptospirosis in California sea lions (*Zalophus californianus*) stranded along the central California coast, 1981–1994. *Journal of Wildlife Diseases* 32: 572–580.
- HEATH, S. E., AND R. JOHNSON. 1994. Leptospirosis. *Journal of the American Veterinary Medical Association* 205: 1518–1523.
- HOWARD, E. B., J. O. BRITT, G. K. MATSUMOTO, R. ITAHARA, AND C. N. NAGANO. 1983. Bacterial diseases. In *Pathobiology of marine mammal diseases*, E. B. Howard (ed.). Vol. 1. CRC Press, Inc., Boca Raton, Florida, pp. 69–118.
- KERR, D. A. 1938. Improved Warthin Starry method of staining spirochetes in tissue sections. *American Journal of Clinical Pathology* 2: 63–67.
- LEES, V. W., AND S. P. GALE. 1994. Titers to *Leptospira* species in horses in Alberta. *Canadian Veterinary Journal* 35: 636–640.
- LUPIDI, R., M. CINCO, D. BALANZIN, E. DELPRETE, AND P. VAEALDO. 1991. Serological follow-up of patients involved in a localized outbreak of leptospirosis. *Journal of Clinical Microbiology* 29: 805–809.
- MATE, M. R. 1978. California sea lion. In *Marine mammals of the eastern north Pacific and Arctic waters*, D. Haley (ed.). Pacific Search Press, Seattle, Washington, pp. 172–177.
- MCILHATTAN, T. J., J. W. MARTIN, R. J. WAGNER, AND J. O. IVERSEN. 1971. Isolation of *Leptospira pomona* from a naturally infected California sea lion, Sonoma County, California. *Journal of Wildlife Diseases* 7: 195–197.
- MEDWAY, W. 1980. Some bacterial and mycotic diseases of marine mammals. *Journal of the American Veterinary Medical Association* 177: 831–4.
- MILLER, D. A., M. A. WILSON, AND G. W. BERAN. 1991. Relationships between prevalence of *Leptospira interrogans* in cattle and regional, cli-

- matic, and seasonal factors. *American Journal of Veterinary Research* 52: 1766–1768.
- NEW, J. C., W. G. WATHEN, AND S. DLUTKOWSKI. 1993. Prevalence of *Leptospira* antibodies in white-tailed deer, Cades Grove, Great Smoky Mountains National Park, Tennessee, USA. *Journal of Wildlife Diseases* 29: 561–567.
- PETERSON, R. S., AND G. A. BARTHOLOMEW. 1967. The natural history and behavior of the California sea lion. *American Society for Mammals Special Publication* 1: 2–20.
- RIEDMAN, M. 1990. *The pinnipeds: Seals, sea lions, and walruses*. University of California Press, Berkeley, California, 439 pp.
- ROLETTO, J. 1993. Hematology and serum chemistry values for clinically healthy and sick pinnipeds. *Journal of Zoo and Wildlife Medicine* 24: 145–157.
- SMITH, R. D. 1995. *Veterinary clinical epidemiology: A problem oriented approach*. 2nd Edition. CRC Press Inc., Boca Raton, Florida, 279 pp.
- SMITH, A. W., R. J. BROWN, D. E. SKILLING, AND R. L. DELONG. 1974. *Leptospira pomona* and reproductive failure in California sea lions. *Journal of the American Veterinary Medical Association* 165: 996–998.
- _____, _____, _____, H. L. BRAY, AND M. C. KEYES. 1977. Naturally-occurring leptospirosis in northern fur seals (*Callorhinus ursinus*). *Journal of Wildlife Diseases* 13: 144–147.
- SWART, K. S., K. CALVERT, AND C. MENEY. 1982. The prevalence of antibodies to serovars of *Leptospira interrogans* in horses. *Australian Veterinary Journal* 59: 25–17.
- SWEENEY, J. C., AND W. G. GILMARTIN. 1974. Survey of diseases in free-living California sea lions. *Journal of Wildlife Diseases* 10: 370–376.
- THIERMANN, A. B. 1982. Experimental leptospiral infections in pregnant cattle with organisms of the Hebdomadis serogroup. *American Journal of Veterinary Research* 43: 780–784.
- TIZARD, I. 1987. *Veterinary immunology: An introduction*. 3rd Edition. W. B. Saunders Company, Philadelphia, Pennsylvania, 401 pp.
- VEDROS, N. A., A. W. SMITH, J. SCHONEWALD, G. MIGAKI, AND R. C. HUBBARD. 1971. Leptospirosis epizootic among California sea lions. *Science* 172: 1250–1251.

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