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EVIDENCE OF *LEPTOSPIRA INTERROGANS* INFECTION IN CALIFORNIA SEA LION PUPS FROM THE GULF OF CALIFORNIA

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ABSTRACT: Forty-two urine and 96 blood and serum samples were obtained from California sea lion (*Zalophus californianus)* pups from the Gulf of California during the 2000 reproductive season. Antibody prevalence to 13 serovars of *Leptospira interrogans* was determined by microagglutination tests (MAT); presence of pathogenic leptospires was detected by polymerase chain reaction (PCR). Samples with antibody titers ≥1:25 or 115 bp fragments on ethidium bromide-stained 1.5% agarose gels were considered positive. Antibody prevalence was 54% overall with highest prevalence against serovar cynopteri (50% of all positive reactions). Highest antibody titers (1:50) were detected against serovars cynopteri and pomona. Polymerase chain reaction products were observed in two of 42 urine samples, six of 96 blood samples, and one of 96 serum samples. Presence of PCR products in blood and serum was demonstrated in pups that were seronegative. Kruskall-Wallis tests and corresponding post hoc Tukey tests (α=0.05) showed that prevalence of leptospirosis was significantly different among all rookeries. The high seroprevalence (54%), low antibody titers (maximum 1:50), absence of pups showing clinical signs indicative of the disease, and lack of recent reports of increased mortality of sea lions in the Gulf of California are suggestive of the presence of enzootic host-adapted serovars. Crowding in rookeries as well as the presence of bats and rodents on some of the islands may explain infection by *L. interrogans* (sensu lato) and some of the differences in seroprevalence among reproductive rookeries.

Key words: Leptospirosis, microagglutination test, polymerase chain reaction, sea lion, serologic survey, *Zalophus californianus.*

INTRODUCTION

In spite of decades of intense study on the biological agents that compose marine communities, the dynamics of infectious diseases and their ecologic and evolutionary impacts in marine ecosystems are poorly understood, even though they can affect species of economic and ecologic value, and may cause mass mortalities or negatively affect abundance and reproduction of host populations (Harvell et al., 1999). This is a consequence of the scant baseline data available needed to understand the epidemiology of infectious agents in marine populations.

Since a mortality event in 1970, free-ranging California sea lions (*Zalophus californianus*) from the coast of California and Oregon (USA) have been affected by leptospirosis outbreaks at regular intervals (Vedros et al., 1971; Dierauf et al., 1985; Gulland et al., 1996), and leptospirosis has been implicated as a cause of premature parturition in California sea lions (Gilmartin et al., 1976).

Leptospirosis is caused by a spirochete, *Leptospira interrogans* (sensu lato), that enters susceptible hosts via cutaneous abrasions and mucosal membranes in contact with body fluids such as urine, blood, and genital secretions (Heath and Johnson, 1994). In spite of the frequency of

these events, the source of infection and means of transmission of leptospirosis in free-ranging sea lions remain unknown (Gulland et al., 1996; Gulland, 1999).

To date, no mass strandings or mortality events due to leptospirosis have been reported in California sea lions in Mexico. Serologic, histopathologic, and clinical findings in some individuals, however, suggest occurrence of leptospirosis in reproductive rookeries from the Gulf of California, but the organism has never been cultured or demonstrated in any sea lions (Acevedo-Whitehouse, 1999, 2001; Godínez et al., 1999).

The California sea lion is the most abundant and widely distributed pinniped species in Mexico and is the only one to permanently inhabit the Gulf of California (Aurioles-Gamboa, 1993). Due to the ecological importance of this species and the impact that outbreaks of leptospirosis could have on their population and to the risk of transmission of this disease to residents of local fishing communities and tourists, both known to have sporadic contact with sea lions, it is important to determine the prevalence of leptospirosis in rookeries from the Gulf of California.

This paper determines the prevalence of antibodies and leptospiral DNA in California sea lion pups from the Gulf of California in 2000. To our knowledge, this is the first definitive indication of *Leptospira interrogans* (sensu lato) infection in free-ranging California sea lions from the Gulf of California.

MATERIALS AND METHODS

Ninety-six blood and 42 urine samples were collected from free-ranging, clinically normal California sea lion pups (1–50 days old) during the 2000 breeding season at seven different reproductive rookeries from the Gulf of California: Los Islotes (24°35.3′N, 110°23′W), San Pedro Mártir Island (28°22.5′N, 112°21′W), San Esteban Island (28°43′N, 112°36′W), Angel de la Guarda Island (29°34′N, 113°33′W), Granito Island (29°33′N, 113°32′W), and Roca Blanca Island (28°5′N, 113°26′W) (Bourillón et al., 1988) (Fig. 1).

Weight was measured by placing the pup in a bag of known weight hanging from a spring scale (accuracy ±0.5 kg). Animals were restrained and anesthetized with isofluorane (Abbott Laboratories, Chicago, Illinois, USA) for sample collection. Urine samples (2–20 ml) were obtained by manual expression of the urinary bladder and collection of contents into sterile wide-mouth containers (Acevedo-Whitehouse, 2001). Blood samples were obtained by venipuncture of the jugular vein using an 18 Gx38 mm needle, in sterile vacuum tubes (Vacutainer®, Becton Dickinson, Franklin Lakes, New Jersey, USA). Whole blood samples (1.5–2 ml) were placed in tubes with ethylenediaminetetraacetic acid (EDTA), another 7–10 ml were placed in tubes for serum separation, allowed to clot at room temperature, centrifuged for 15 min at 3,000 G and separated. All samples were stored at −20 C.

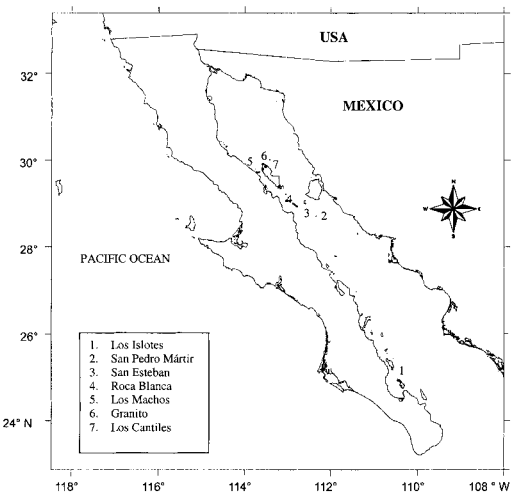

FIGURE 1. California sea lion reproductive rookeries in the Gulf of California.

Rookeries were searched for stagnant pools. Five pools were chosen at random at all rookeries where stagnant seawater pools were present. Temperature, salinity, and pH of the water were instantly determined with a digital environmental thermometer (Banrnant100®, Davis Instruments, Enterprise, Oregon, USA), a salinity refractometer (A366ATC, VeeGee Scientific, Kirkland, Washington, USA), and a potentiometer (Markson 90, Markson Science Inc., Phoenix, Arizona, USA). Three replicate samples of seawater from each pool were collected in 250 ml sterile containers and stored at −20 C.

Polymerase chain reaction (PCR) was performed on all blood, serum, and urine samples. For DNA extraction, three replicates of 200 μl of each sample were processed according to

GeneReleaser® protocols (BioVentures, Inc. Murfreesboro, Tennessee, USA). The 23S ribosomal oligonucleotide primers F1 (5′-GAACTGAAACATCTAAGTA-3′) and Ri (5′-CAGCGAATTAGATCTG-3′) described previously by Woo et al. (1997) were used for DNA amplification. Each 50-μl PCR reaction contained 200 ng/μl of each primer, 0.05 mM dNTP, 5 μl of 10X reaction buffer (0.5 mM Tris-HCl, 0.075 mM $MgCl_2$, 2.5 mM KCl, pH 7.5), 1 unit of Taq DNA polymerase (Roche, Mannheim, Germany), and 5 μl of target DNA. The PCR cycle was as follows: initial denaturation at 94 C for 5 min, followed by 25 cycles of denaturation at 94 C for 1 min, annealing at 44 C for 1 min, extension at 72 C for 2 min, and a final cycle of extension at 72 C for 7 min (Woo et al., 1997).

Reference DNA from strains of *Leptospira interrogans* icterohaemorrhagiae and *L. biflexa* patoc1, obtained from the Unit of Molecular and Medical Bacteriology of the Pasteur Institute (France) were used as positive and negative controls, respectively. The amplification products (5 μl of the reaction mixture) were analyzed by submarine electrophoresis using 1.5% agarose gel stained with ethidium bromide.

Primer sequences were aligned using Megalign (Megalign software, DNA Star Inc, Madison, Wisconsin, USA) in order to compare the 23SrDNA reported for pathogenic and non-pathogenic strains of *Leptospira* (Woo et al., 1997). In addition, sequences of potential contaminant bacteria like *Bacillus subtilis, Escherichia coli,* and *Pseudomonas* spp. were included in the alignment.

Standard microscopic agglutination tests (MAT) were preformed according to Myers (1985) on all serum samples. Live strains of 13 serovars of *L. interrogans* were used as antigens: autumnalis, bataviae, canicola, castellonis, cynopteri, grippotyphosa, hardjo, icterohaemorrhagiae, pomona, pyrogenes, sejroe, tarassovi, and wolffi. All serovars are part of the collection of the Departamento de Microbiología e Inmunología (Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México). The collection was previously obtained from the former Zoonoses Panamerican Center (OPS/OMS), Buenos Aires, Argentina. Antibodies to these serovars were previously described for California sea lions in the Gulf of California (Godínez et al., 1999). Positive tests were reported as the greatest serum dilution at which serum showed a reaction. Although titers of 1:100 are typically considered to indicate infection with clinical disease (Gulland et al., 1996), in this study titers as low as 1:25 were considered as positive agglutination reactions. Choice of MAT cut-off is arbitrary, and titers of 1:20 and 1:24 have been previously used as positive reactions (Hathaway et al., 1978; Godinez et al., 1999). Amplification of 115 bp PCR fragments in urine, blood or serum was considered positive for presence of leptospiral DNA.

Differences in the seroprevalence among rookeries were investigated by Kruskall-Wallis and post hoc Tukey tests (Zar, 1999). Chi-square tests in three-dimensional contingency tables were used to determine independence among rookery, sex, and weight of the pups as a measure of body condition. Partial independence between seroprevalence, sex, and rookery was determined by a posteriori χ^2 tests (Zar, 1999). Correlations were used to determine that the differences in seroprevalence among rookeries and number of serovars that generated agglutination reactions were not due to the sample size of each rookery.

Significance of all statistical tests was determined at $\alpha=0.05$.

RESULTS

Fifty-two (54%) of 96 pups were seropositive on MAT. Prevalence of antibodies was significantly different among the seven rookeries ($H_c=14.4045$, $\upsilon=6$, $P<0.05$) (Table 1) and showed a latitudinal trend, being significantly highest at Granito (90%) ($q=7.23$, $k=7$, $P<0.05$), the northernmost rookery included in this study, and lowest at Los Islotes (20%), the southernmost rookery of the Gulf of California (Fig. 1). Prevalence of antibodies appeared to be independent of the pup's weight ($\chi^2=2.256$, $\upsilon=8$, $P>0.05$), but was significantly higher in female pups than in males ($H_c=13.617$, $P<0.05$). Highest dilution at which agglutination was observed was 1:50. No samples were positive at further dilutions. Highest antibody titers were observed for serovar cynopteri (26% of all seropositive pups) and pomona (2% of all seropositive pups).

Twenty-three serum samples had agglutination reactions against more than one serovar; seven, four, five, and seven samples reacted against two, three, four, and more than five serovars, respectively. The serovar which showed most reactions was cynopteri, accounting for 50% of all agglutination reactions, followed by hardjo

TABLE 1. Results of microagglutination tests (MAT) and polymerase chain reaction (PCR) amplification in samples from sea lion pups of the Gulf of California.

	Seroprevalence		PCR amplification of leptospiral DNA					
			Blood		Serum		Urine	
Rookery	%	*n*	Positive samples	Samples examined	Positive samples	Samples examined	Positive samples	Samples examined
Los Islotes	20	10	0	10	0	10	0	5
San Pedro Mártir	30	10	1	10	0	10	0	7
San Esteban	33	15	0	15	0	15	0	3
Roca Blanca	70	10	0	10	0	10	0	8
Los Machos	73	11	2	11	1	11	2	7
Granito	90	20	3	20	0	20	0	6
Los Cantiles	45	20	0	20	0	20	0	6
Total	54	96	6	96	1	96	2	42

(18%), and pomona (13%). Reactions to serovars canicola and bataviae were observed in conjunction with pomona; grippotyphosa in conjunction with cynopteri; and icterohaemorrhagiae and pyrogenes in conjunction with hardjo.

We observed 115 bp PCR products in two of 42 urine samples, six of 96 blood samples, and one of 96 serum samples (Table 1; Fig. 2). During alignment, homology was observed only with the reported sequences for *Leptospira interrogans* (sensu lato), and all other compared sequences, including non-pathogenic leptospires, were significantly non-specific.

No pathogenic leptospiral DNA was found in any of 75 water samples tested from stagnant-water pools at Los Islotes, San Esteban, Roca Blanca, Granito, and Los Cantiles. Pool water temperature ranged from 23.2–36.8 C, salinity from 35–44 and pH from 7.3–7.8.

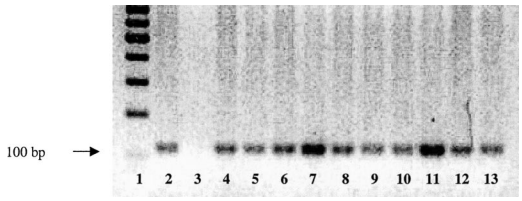

FIGURE 2. PCR amplification of 115 bp *L. interrogans* products in urine, blood, and serum samples of sea lion pups (Lanes 4 to 13), lane 3: *L. biflexa* control DNA, lane 2: *L. interrogans* control DNA, lane 1: 100 bp molecular weight marker (LowLadder, Sigma-Aldrich Corp., USA).

DISCUSSION

Pups that had pathogenic leptospiral DNA in blood or urine were considered infected with leptospires (Van Eys et al., 1989; Merrien et al., 1992). Antibody production is dependent on the host's ability to develop an immune response that may be influenced by sex, genetics, and nutritional status (Gulland, 1997). Because the sea lion pups were free-ranging, antibody titers were not due to vaccination responses, but could be due to maternal transmission of antibodies to the pup or to prior exposure of the pup to infection (Michna, 1970; Gulland, 1997).

The low prevalence of leptospiruria detected in this study may be a consequence of intermittent shedding (Michna, 1970; Heath and Johnson, 1994) or of the detection limit of the PCR test used, as PCR detects ≥50 leptospires per ml of urine (Wagenaar et al., 2000). Pups with leptospiruria had antibody titers of ≥1:50 against *L. interrogans* (sensu lato) and pups with leptospiremia had no detectable titer. These findings concur with the pathogenesis of leptospirosis in domestic animals in which antibody production is not detectable during the initial leptospiremic stage of the infection (Michna, 1970) but can be found during leptospiruria, a stage that occurs later in infection (Thiermann, 1982).

Contrary to other reports, we found

PCR detection of pathogenic leptospiral DNA to be more sensitive in blood than in serum samples. Leptospiral DNA fragments in blood were observed in all leptospiremic pups but only one had detectable leptospiral DNA in serum. Gravekamp et al. (1993) reported that erythrocytes inhibit amplification of pathogenic DNA segments during PCR and suggested that serum is a more appropriate sample for the diagnosis of leptospirosis. In this study, the DNA extraction method allowed the use of up to 200 μl of whole blood without the interference of inhibitory compounds during amplification (Dawson and Harris, 1995). It is probable that sequestering of leptospires in the blood-clot (Gravekamp et al., 1993) accounted for decreased sensitivity in serum samples.

The route of infection of California sea lions by leptospires is unknown. Transmission of leptospires during lactation is unlikely due to the presence of lytic lipids in maternal milk (Stalheim, 1965). Pups may have become infected in utero (Thiermann, 1982), or during the first days of life at the rookery by contact with urine from infected sea lions or other reservoirs (Heath and Johnson, 1994; Gulland et al., 1996). An infected host may eliminate up to 10^5 leptospires/ml urine during the first weeks of infection (Heath and Johnson, 1994), and it has been reported that sea lions eliminate leptospires in the urine for up to 154 days after infection (Dierauf et al., 1985). Maintenance of leptospirosis in California sea lions could be enhanced by crowding in rookeries and contact with urine. Leptospiral DNA was not found in water samples from stagnant seawater pools at the rookeries. Although the presence of pathogenic leptospires in the water below the detection limit of PCR cannot be ruled out, the temperature and salinity levels recorded at the pools would compromise the viability and infectivity of *L. interrogans* (sensu lato) (Michna, 1970; Ellinghausen, 1973). Thus, stagnant pools are not likely a source of infection or a method of maintenance of leptospirosis in California sea lions at rookeries from the Gulf of California.

A previous study reported highest prevalence to serovar hardjo (22%) and cynopteri (14%) in sea lions from the Gulf of California, and higher titers of up to 1:320 (Godínez et al., 1999). We observed 50% of all positive agglutination reactions to serovar cynopteri and both pups with leptospiruria had antibodies to this serovar; whereas prevalence to serovar hardjo only accounted for 6% of all agglutination reactions. These differences may be due to differences in exposure of animals between years, or differences in immune status of the host. Agglutination reactions to serovars autumnalis, bataviae, canicola, castellonis, grippotyphosa, icterohaemorrhagiae, pyrogenes, sejroe, tarassovi, and wolffi could be due to cross-reactions to serovars used for MAT, although they could reflect exposure to other leptospires (Myers and Coltorti, 1978; Hanson, 1982; Gulland et al., 1996).

The prevalence of leptospiral serovars in a population reflects the prevalence of serovars in reservoirs (Heath and Johnson, 1994). The fishing bat (*Myotis vivesi*) as well as endemic and introduced rodents (*Peromyscus* spp., *Mus musculus, Rattus rattus,* and *Rattus norvergicus*) have been reported on some of the islands (Maya, 1968; Lawlor, 1983). Rodents and bats may be reservoirs of leptospires (Michna, 1970; McCoy, 1974; Thiermann, 1981; Hanson, 1982). Although it has not been established if the sea lion rookeries are used by these potential reservoir species, this information would be useful in understanding the epidemiology of leptospirosis in sea lions in the Gulf of California.

The presence of potential mammalian reservoirs of leptospires on the islands might explain the presence of *L. interrogans* but does not fully explain differences in the seroprevalence among rookeries. Sea lions move between northern and southern rookeries. Juvenile sea lions branded as pups at Los Islotes have been seen at Granito, San Pedro Mártir and San

Esteban (Aurioles-Gamboa, pers. comm.). Due to the proximity of the northern rookeries, exchange of animals among these is probably common. If traveling animals become infected with leptospires they could move infection among rookeries. The latitudinal increase in seroprevalence could reflect movement of animals among northern rookeries.

High antibody prevalence (54%), low antibody titers, absence of pups showing clinical signs suggestive of leptospirosis, and the lack of increased mortality of sea lions in the Gulf of California, indicate enzootic leptospiral infection caused by host-adapted serovars (Heath and Johnson, 1994). Due to the relative separation of sea lions between the northern Pacific and Gulf rookeries (Aurioles-Gamboa et al., 1983; Maldonado et al., 1995), it is probable that the sea lion population from the Gulf of California has not had contact with infected sea lions from the northern Pacific.

This paper demonstrates the presence of pathogenic leptospiral DNA in sea lions in the Gulf of California. In order to assess the impact of enzootic leptospirosis and possible epizootic events of this disease in sea lions from the Gulf of California, distribution of pathogenic leptospires in all age and sex classes of sea lions must be described; sources of infection, and means of transmission and persistence of infecting serovars must be determined; and the nature and duration of sea lion immunity to pathogenic leptospires must be established.

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