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Short Communication

Serologic studies of bacterial zoonoses in free-living sea lion pups *Zalophus californianus*, in the Gulf of California, Mexico

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ABSTRACT. This study aimed to investigate the prevalence rate of brucellosis, leptospirosis, and chlamydiosis in the offspring of free-living sea lions (*Zalophus californianus*) in the Mexican Gulf of California. The work was carried out across six islands where reproduction takes place. Sixty-one blood samples were collected from seemingly healthy sea lions from six to eight weeks old. For diagnosis, serum was obtained by venae cavae puncture. In the search for smooth *Brucella* spp., based on phenotypic characterization, antigens were detected employing an antigen test for *Brucella abortus*, strain 1119-3. In the case of leptospirosis, the serological diagnosis was carried out by microscopic agglutination to identify 12 *Leptospira interrogans* serotypes. Lastly, *Chlamydia abortus* was identified using commercial ELISA (ID Screen®). All 61 serum samples presented negative results for the *B. abortus* antigen and the *C. abortus* ELISA. The serological diagnosis for leptospirosis showed that 29 out of 61 sera were negative (47.5%) for all serotypes, and 32 out of 61 were positive (52.5%) for at least one serotype. After studying the three diseases, results suggest that the presence of antigens against *Leptospira* is a potential threat to the protected sea lions from the Gulf of California.

Keywords: *Zalophus californianus*; brucellosis; leptospirosis; chlamydiosis; Gulf of California; Mexico

The Californian sea lion (*Zalophus californianus*) is a worldwide-protected marine mammal. The species is distributed throughout the west of the Northern Hemisphere; it is among the most abundant pinnipeds in Mexico and a permanent resident of the Gulf of California (Aurioles-Gamboa 1993). Nevertheless, the population in the Gulf of California has decreased over the past decades (Underwood et al. 2008). These marine mammals are held under special protective legislation, supported by Official Mexican Law NOM-059-SEMARNAT-2010 (SEMARNAT 2010).

Among the causes that have affected their population is the high pressure exerted by fishing, which raises the incidental capture of birds and marine mammals (Underwood et al. 2008).

Brucellosis has been searched for in sea lions in various locations. Some countries have demonstrated their presence by identifying antigens and detecting their DNA with PCR (Avalos-Téllez et al. 2014). In Alaska, *Brucella* spp. was detected in Steller sea lions (*Eumetopias jubatus*) (Esquible et al. 2019). However, other countries, such as New Zealand, have failed to identify whether brucellosis affects sea lions (Roe et al. 2010). A previous study at Isla San Esteban in the Gulf of California, during June and July 2011, examined 22 newborn female Californian sea lions during the period

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of lactation, to determine exposure to *Brucella* spp. Culture of blood, vaginal discharge, and milk samples revealed negative results, but serological evidence proved that five sea lions (22.7%) had been exposed to *Brucella* spp. (Avalos-Téllez et al. 2014).

Brucella ceti and *B. pinnipedialis* are marine mammals' most common infectious diseases worldwide (Abe et al. 2017, Ohishi et al. 2018). San Esteban, in the Gulf of California, during June and July 2011, examined 22 newborn female Californian sea lions during lactation to determine exposure to *Brucella* spp. Culture of blood, vaginal discharge, and milk samples revealed negative results, but serological evidence proved that five sea lions (22.7%) had been exposed to *Brucella* spp. (Avalos-Téllez et al. 2014).

Leptospirosis is a systemic bacterial infection that affects domestic animals, wildlife, and humans. Many wild animals act as hosts for *Leptospira*. Therefore, the transmission of the illness is linked to multiple factors throughout the animal-human-ecosystem interface (Petrakovsky et al. 2014, Vieira et al. 2018). Leptospirosis is the second most common reason for diseased and stranded sea lions along the coast of California (Greig et al. 2005).

Chlamydiae are intracellular pathogens known to cause illness in humans and animals. More than 400 host species have been documented worldwide; most are wild animals (Burnard et al. 2016). The more we learn about the genetic diversity of this group of pathogens, the greater the evidence that these bacteria can infect a wider range of animal hosts than previously thought.

Research on *Chlamydia* spp. in sea lions is rare. A previous study on Steller sea lions in Alaska found *Chlamydia* spp. (Esquible et al. 2019). Further evidence that justified the present research is the findings of *Chlamydia psittaci* in water birds from New Zealand and water birds kept in a wildlife rescue center from the west of France, in the North Atlantic (Aaziz et al. 2015, Soon et al. 2021). Moreover, previous reports (Herrmann et al. 2000, Pantchev et al. 2009, Szymanska-Czerwinska et al. 2017, Origlia et al. 2019) identified *Chlamydia abortus* avian strains across wild and domesticated bird species. Nevertheless, the role of the strain in the transmission of avian infections is not clear yet.

This study aimed to investigate the prevalence rate of brucellosis, leptospirosis, and chlamydiosis antigens in the offspring of free-living sea lions in the Mexican Gulf of California.

Under the Mexican Government authorization NºSGPA/DGVS/01798/21, sea lion pups were sampled during July 2021 in the islands of the Gulf of California; Coloradito (30°02'55.13"N, 114°29'25.06"W), Granito (29°33'51.06"N, 113°32'34.70"W), Los Cantiles (29°31'25.34"N, 113°28'29.55"W), Machos (29°17'8. 34"N, 113°29'51.66"W), Rasito (28°50'12.84"N, 112°59'56.01"W) and El Partido (28°53'36.01"N, 113°02'55.13"W) (Fig. 1). Sixty-one samples were obtained, 10 at each of the first five islands and 11 at El Partido.

The purpose of the annual monitoring of the pups is to gather information about their health status during the births of that season, accomplished by capturing the pups for morphometric measurements and taking biological samples to assess the prevalence of diseases that have already been reported in these colonies and that may affect the population. This process is not carried out for larger animals as it is not the goal of the annual monitoring and requires specialized equipment, such as rifles and darts, which necessitates obtaining additional permits. Invasive blood sampling was minimized when collected from pups in the rookeries of the six islands in the Gulf of California. Blood samples were collected from 61 manually restrained lactating sea lion pups, six to eight weeks old, from the jugular vein using 18 gauge, 38 mm needles. Samples were kept in separate sterile serum tubes and centrifuged at 8000 rpm for 10 min. After refrigeration, the serum aliquots were transported to the laboratory and frozen at -20% until analyzed. Anesthesia was supplied via inhalation with 5% isoflurane and maintained at 3% (Haulena & Heath 2001).

Antigens against smooth *Brucella* spp. were detected using the *B. abortus* strain 1119-3 test, with 8% cellular density (Alton et al. 1988). To detect antibodies against pathogenic *Leptospira* serotypes (Icterohaemorrhagiae, Bratislava, Pyrogenes, Grippotyphosa, Canicola, Pomona, Wolffi, Hardjo, Tarassovi, and three national isolates Palo alto (Icterohaemorrhagiae), H-89 (Hardjo), and Portland vere (Canicola), serum samples were tested for microscopic agglutination (MAT), according to the reagents and method specified by the National Institute of Forestry, Agriculture and Livestock Research (INIFAP, by its Spanish acronym), Mexico (NOM-029-SSA2, 1999).

Sera were diluted at 1:50 for a screening test, and 50 µL of the 12 serotypes were added to 96-well flat-bottom microtiter plates (Nunc, Maryland, USA). The plate was gently stirred and incubated for 1 h at room

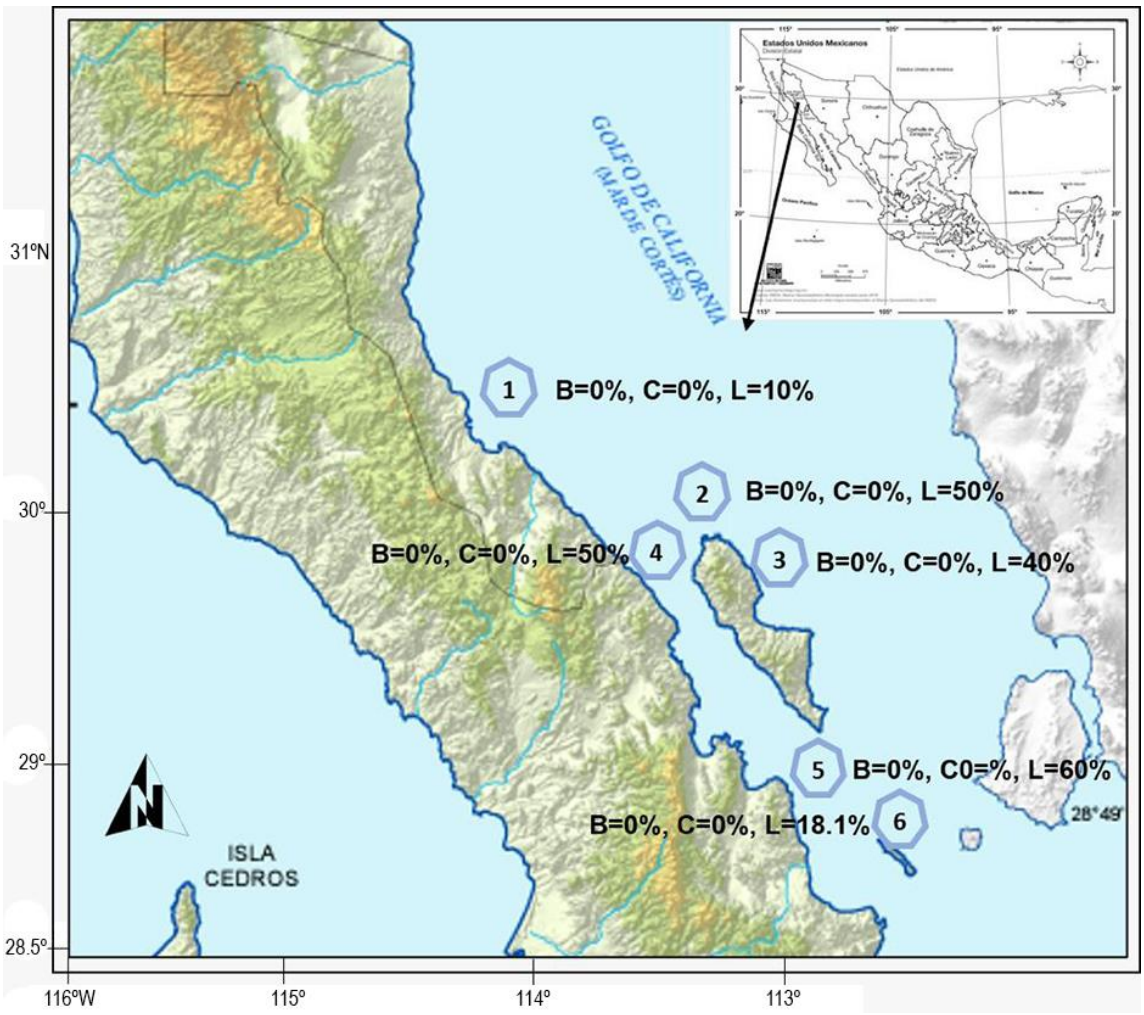

Figure 1. Prevalence for brucellosis (B), chlamydiosis (C), and leptospirosis (L) in 61 sampled free-living sea lions in the Gulf of California, Mexico. Sampling sites. 1: Coloradito, 2: Granito, 3: Cantiles, 4: Machos, 5: Rasito, 6: Partida.

temperature in a humid chamber and analyzed with dark-field microscopy (Carl Zeiss, Germany). Sera were then titrated by serial dilutions from 1/50 to 1/800 for each serotype, searching for micro-agglutination. The final titer represents the maximum dilution by which a grade-two agglutination was observed. Titers higher or equal to 1:100 were considered positive. Negative control was included for each serovar.

Antigens against *Chlamydia* spp. were detected using an ELISA commercial Indirect Multi-species kit, which identifies *C. abortus* antibodies in serum utilizing a synthetic antigen obtained from a protein in the external membrane (ID Screen®).

All 61 sea lions sampled at the six islands presented negative results to the antigen test for smooth brucellosis (Alton et al. 1988). Furthermore, according to diagnoses with ELISA, all sea lions were negative for *C. abortus* (Fig. 1).

Based on the serological diagnosis, leptospirosis results proved negative for all serovars in 29 out of 61 (47%) sea lions and positive for at least one serovar in 32 out of 61 (52%) sea lions (Table 1). All 61 sea lions were negative for Wolffi, Tarassovi H-89, and Palo alto serovars (Fig. 1).

In Coloradito Island, two sea lions were Icterohaemorrhagiae positive, one with titer 1:200 and the other with titer 1:400; and nine sea lions proved negative for all serovars.

In Los Cantiles Island, one sea lion was Hardjo positive, with a titer of 1:800; another was Pomona positive, with a titer of 1:200; two sea lions were Icterohaemorrhagiae positive, with a titer of 1:400; and six sea lions proved negative for all serovars.

In Granito Island, one sea lion was Bratislava positive, with titer 1:800, and Pyrogenes and Grippo-

Table 1. Results for leptospirosis serological diagnosis in 61 sampled free-living sea lions in the Gulf of California, Mexico. Microscopic agglutination was used to identify antigens against 12 serovars. Titers of 1:200 or higher were considered positive results.

Island	Samples	Negative	Positive	Serotypes identified
Rasito	10	4	6	Canicola, Grippotyphosa, Pomona.
Machos	10	5	5	Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pyrogenes, Portland vere.
Granito	10	5	5	Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pyrogenes
Cantiles	10	6	4	Hardjo, Icterohaemorrhagiae, Pomona
Partido	11	9	2	Bratislava, Grippotyphosa, Pyrogenes
Coloradito	10	9	1	Icterohaemorrhagiae
Total	61	29 (47.5%)	32 (52.5%)	

typhosa positive, both with titers 1:200; two sea lions were Bratislava positive with titers 1:200; one sea lion was Bratislava positive with titer 1:400, and Pyrogenes and Canicola positive, both with titers 1:200: one sea lion was Icterohaemorrhagiae and Pyrogenes positive, both with titers 1:200, and Bratslava positive with titer 1:400, and five sea lions proved negative for all serovars.

In Machos Island, two sea lions were positive for Icterohaemorrhagiae and Bratislava, both with titers 1:200. Additionally, one of the sea lions was positive for Canicola serotype, with titer 1:400. One sea lion was Pyrogens and Grippotyphosa positive, both with titers 1:200; one sea lion was Bratislava positive, with titer 1:800; and five sea lions showed negative results for all serovars.

In Rasito Island, two sea lions were positive for the Grippotyphosa serotype, with titer 1:200; three sea lions were positive for the Canicola serotype, one with titer 1:400, and two with titers 1:200; one sea lion was positive for Canicola and Pomona serotypes, both with titers 1:400; and four sea lions were negative for all serovars.

Finally, in El Partido Island, one sea lion was Grippotyphosa positive, with titer 1:600; another was Bratislava and Pyrogens positive, both with titer 1:200; and eight sea lions proved negative for all serovars.

The present study did not detect *Brucella* spp. antigens in any sample from the 61 lactating sea lion pups, probably because evidence of infections by *Brucella* spp. is mostly found in animals that have developed sexual maturity. Still, antigens could have been detected because mothers can transmit them to pups through colostrum or by way of their close attachment -a mother-pup behavior demonstrated in previous studies of sea lions from the Gulf of California (García-Aguilar & Aurioles-Gamboa 2003).

There is a previous history of *Chlamydia* spp. in sea lions. However, it was not carried out in sea lions, but in Steller's sea in Alaska, where the sampling of 47% of premature pups, 32% of spontaneous abortions, 11% of newborns, and 11% intrauterine fetuses. The macroscopic findings by necropsy and histology were confirmed by PCR (Esquible et al. 2019).

Leptospirosis can be spread by direct or indirect contact with urine from infected animals or by urine-contaminated water reservoirs of domestic and wild animals (Monahan et al. 2009, Monroy-Díaz et al. 2020). The route of transmission of leptospirosis among sea lions has not been determined yet. However, oral transmission is very likely, because it is the predominant route in domestic and other wild animals. Contagion probably occurs at little pools formed by sea water and sea lion urine, which accumulate in places where sea lions of all ages gather. This may constitute an excellent niche for bacterial cultures growing within the species' habitat and allow the spread among other animals.

A previous study by our group also focused on sea lion pups on the islands in the Gulf of California (Avalos-Tellez et al. 2016) and found serological antigens for at least one *Leptospira* serotype in all the sampled sea lions. Moreover, at least one sea lion was positive for one out of 11 serovars among the 12 serotypes specified in the MAT. In contrast, the present study found 32 out of 61 (52.5%) sea lion pups positive to at least one of the serotypes, and agglutination was displayed by 7 out of 11 serovars specified in the MAT. Over the past five years, the lack of control measures has prevented a conclusion about the reasons for the decline in the number of positives. In addition, no significant die-off among the sea lion population has been registered in Mexico, as observed in previous years on the coast of the USA (Greig et al. 2005).

In this study, the 61 sampled sea lions were clinically healthy and showed no symptoms of leptospirosis. Nevertheless, some studies indicate that sea lions can be asymptomatic carriers (Cameron et al. 2008, Prager et al. 2013). Within their first months of life, the pups present direct contact with feces and urine that may contain *Leptospira* because they spend the longest time between the resting rocks; as described in previous studies, hatchlings present high titers of antibodies of different serovars of *L. interrogans* (Avalos-Téllez et al. 2016). Therefore, as the hatchlings are smaller and easier to handle, they can provide information to know the seroprevalence of *Leptospira* within the reproductive colonies.

The maximum antibody titer identified in this study was 1:800, the highest dilution used for analysis. The results of the titers are high but still below the levels recognized in sea lions by other authors who used higher dilutions (Pedernera 2004, Avalos-Téllez et al. 2016).

MAT findings of positive Pyrogenes and Wolffi serotypes coincide with previous reports from the Gulf of California (Pedernera 2004), as does the Pomona serotype (Avalos-Téllez et al. 2016).

There are correlations between the present study and the one carried out by this research team in 2016. For example, specific serovars coincide across different islands: in Granito Island, sea lions showed positive MAT results to Bratislava, Pyrogenes, Canicola, Icterhaemorrhagiae, and Grippotyphosa; in El Partido Island, sea lions showed positive MAT results to Grippotyphosa, Bratislava, and Pyrogenes; and in Coloradito Island, sea lions showed positive MAT results to Icterohaemorrhagiae serotype. It is relevant to point out that sampling in 2016 showed a higher number of serovars across the four islands than the values identified in the present study (Avalos-Téllez et al. 2016).

In contrast, most samplings showed negative MAT results in the Coloradito and El Paso islands. Both are very small islands, rarely visited by humans. In Coloradito, there was one positive result out of 10 samples, and in El Partido Island, there were two positive results out of the 11 sampled sea lions. Fishermen cannot unload on El Partido Island; it is prohibited.

The cause behind recurring epidemics in sea lion populations is unknown. They could be caused by external infections after direct contact with carrier animals, wild or domestic. They could also occur due to epidemic cycles associated with the acquired immunity of the population.

Feral cats are an introduced species that cohabit with sea lions in Machos and Cantiles. In 2016, analyzed the sera of five cats that showed positive MAT results to various serotypes: Automnalies 1/50, Batavie 1/(50), Bratislava 1/(100)-1/(200), Canicola 1/200-1/400, Celledoni, 1/50 to 1/800, Gripptiphosa 1/100); and Pomona 1/50- 1/100. As in cats, MAT results in Isla Machos revealed that sea lions were positive for Bratislava, Canicola, Grippotyphosa, and additionally to Portland vere, Icterohaemorrhagiae, and Pyrogenes. These results confirm that the pathogen circulates on the sea lion colonies' islands (Ortiz-Alcaraz et al. 2017).

The current study sampled islands that could not be sampled in 2016 (Avalos-Téllez et al. 2016). For example, in Rasito Island, sea lion MAT results were positive for Grippotyphosa, Canicola, and Pomona serovars. This island is small and surrounded by cliffs, which reduces contact with humans and other mammals. Therefore, the remaining hypothesis is that birds or other animals sharing the marine ecosystem transport the pathogen.

The data collected herein for the three infectious diseases suggest that *Leptospira* is the only bacteria potentially harming the health of the sea lions protected in the Gulf of California. Although the number of positive cases among sea lions is higher than in 2016 (Avalos-Téllez et al. 2016), the behavior of this pathogen has proven to be cyclical in the coastal USA. Therefore, an environmental condition, or an intrinsic condition in the sea lions, could be affecting the presence of the pathogen. Hence, further studies are needed that focus on the interactions between the species and the marine environment or a possible correlation with the growth of human presence (Roe et al. 2010).

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