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# A four-year serological survey of *Leptospira* spp. in stray dogs from northwest Mexico

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## Abstract

The presence of stray dogs in a community represents a significant risk factor in the spread and maintenance of leptospira by acting as pathogen reservoirs and potentially transmitting the bacteria to household dogs and the peri-domestic environment, increasing the risk of zoonotic transmission of the disease. Here, we utilized an indirect ELISA employing recombinant LipL32 protein to detect IgG antibodies to leptospira in serum samples from stray dogs in Mexicali, Northwest Mexico. Serum samples (n = 331) from stray dogs were collected from 2017 to 2020 and tested with the ELISA-LipL32. The results showed an overall seroprevalence of 46.52 % (154/331) over the four years, 46.36 % (51/110) in 2017, 57.95 % (51/88) in 2018, 28.85 % (15/52) in 2019, and 45.68 % (37/81) in 2020. The high prevalence of serum antibodies to *Leptospira* among the stray dog population indicates that Mexicali provides optimal ecological conditions for maintaining and disseminating leptospira throughout the city, increasing the risk of infection to other domestic or companion animals and the human population. Further epidemiological research is necessary to identify the serovars in the region and propose strategies to control the stray dog population and the propagation of leptospira in the Northwest region of Mexico.

***Keywords:*** ELISA; *Leptospira* spp.; Recombinant LipL32; Stray dogs; Zoonoses.

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## Study contribution

This work represents the first report of serological evidence for the presence of pathogenic strains of leptospira in the stray dog population of the municipality of Mexicali, highlighting the need for further research and interventions to control this important zoonotic disease in the Northwest region of Mexico. To address this need, an ELISA-LipL32 system was developed in this study that can be utilized as a diagnostic tool to screen subjects for leptospira infection across various animal species, including humans, and determine the clinical and epidemiological status in our region.

The availability of an ELISA-rLipL32 serological platform was successfully adapted and optimized to obtain an accurate, fast, low-cost serological tool for detecting dogs previously or suspected to be currently infected with pathogenic leptospira strains.

## Introduction

Leptospirosis is a zoonotic infectious disease caused by spirochetes of the genus *Leptospira*.[1] Numerous international health organizations consider leptospirosis as a neglected and ignored disease, with an emerging or reemerging situation in many countries. Leptospirosis is distributed worldwide among the human population and in multiple animal species.[2] With at least thirty recognized serotypes and more than 350 serovars of pathogenic, intermediate, and saprophytic strains, the genus *Leptospira* comprises a group of bacteria that infects more than a million people and produces an estimated 60 000 deaths per year worldwide.[3] The social and economic situation in developing countries, where inhabitants suffer from overpopulation and poor hygienic and sanitary conditions, suggests that the morbidity and mortality rates of leptospirosis are higher than currently reported.

Because of its nature as a neglected disease, the epidemiological situation of leptospirosis in humans, particularly in domestic or companion animals is unknown. The latter is due to the lack of health programs designed for routine screening and detection of the disease or insufficient availability of appropriate and affordable diagnostic tools to meet the demand for this public health problem.[4]

Leptospira infection occurs by direct contact with urine, blood, and tissues of infected animals or indirectly through abrasions, inhalation of aerosols, and contact with soil, water, and food, contaminated with *Leptospira* sp.[5] Leptospirosis produced by *Leptospira interrogans* serovar *Icterohaemorrhagiae* and *Canicola* are considered the main serovars affecting dogs.[6] Dogs are frequently exposed to pathogenic strains of Leptospira and become infected by direct or indirect contact with other domestic or wild-infected animal reservoirs in their immediate outdoor environment. This contamination might lead to an infection that brings leptospira into the household, increasing the risk of zoonotic transmission of the disease between dogs and their owners by direct contact with *Leptospira* ssp. shed in the urine or present in another common contaminated source like water.[7]

Definite diagnosis of leptospirosis requires confirmation of the presence of the bacteria in different body fluids such as urine, blood, cerebrospinal fluid (CSF), or infected tissues. Dark-field microscopy is a direct conventional method for the demonstration of the presence of *Leptospira* ssp. in blood or CSF samples. How-

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ever, this technique has low indices of accuracy and is not recommended as a sole diagnostic procedure for early diagnosis of leptospirosis.[8]

Microbiological isolation of leptospira requires the use of specific culture media, such as the Ellinghausen-McCullough-Johnson-Harris medium, which is inoculated with samples of blood or urine and kept under incubation for up to three months seeking bacterial growth. Again, this procedure is laborious and complicated, and requires high biosafety level facilities and long periods to produce a positive culture, resulting in an impractical procedure for the routine diagnosis of leptospirosis.[4]

Molecular techniques have also been widely used for the diagnosis of leptospirosis with high levels of sensitivity and specificity. Polymerase chain reaction (PCR) techniques are useful to detect *Leptospira* ssp. given the levels of sensitivity and specificity close to 100 %, and the ability to detect minute amounts of bacterial DNA in any given sample.[9] The development and application of quantitative PCR systems resulted in a substantial improvement in the detection of leptospira. This technology has been used as a rapid and reliable diagnostic tool for leptospirosis with better levels of sensitivity and specificity than the microscopic agglutination test (MAT) or microbiological culture, with important implications for the epidemiological surveillance programs and clinical investigations of this disease.[10] PCR techniques also require expensive laboratory equipment, samples are prone to contamination, test protocols require initial standardization and validation, and highly trained personnel.[11]

Serological tests to detect antibodies to leptospira are the most widely used methods for indirect diagnosis of leptospirosis in humans and animals. From these MAT results, the gold standard diagnostic test for leptospirosis, requires highly trained personnel to interpret the results and to handle live strains of pathogenic *Leptospira* ssp. under biosafety conditions available only in reference or research laboratories.[12] During the last decade, important modifications have been made to the serological tests available to improve the quality of the results, one of the most reliable is the use of recombinant LipL32 (rLipL32) protein as antigen for the detection of antibodies specific for pathogenic leptospira strains.[13] The use of the protein LipL32, a highly conserved immunodominant protein expressed only in pathogenic species of leptospira,[14] has been successfully adapted into different serological platforms for the diagnosis of leptospirosis in humans[15] and animals.[16–18]

In Mexico, leptospirosis is classified as a notifiable disease with weekly case reports in humans occurring in the majority of the country's states, however in the last ten years, for the state of Baja California, only 35 human leptospirosis cases have been reported compared with 2 260 cases notified for the rest of the country,[19] these low number of cases probably does not reflect the current epidemiological situation of human leptospirosis for this region. Regarding the epidemiological situation of leptospirosis in dogs or other animal species, Baja California hasn't information available.

Considering the lack of clinical and/or epidemiological data for our region and the importance of leptospirosis as a cause of disease in humans, the objective of this study was to determine the prevalence of anti-leptospira antibodies in stray dogs from the municipality of Mexicali in four years using an ELISA system based on the rLipL32 protein as the capture antigen.

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## Materials and methods

### Ethical statement

The blood samples obtained for this study were collected entirely by personnel from the Mexicali Municipal Animal Control Center (MACC) from dogs scheduled for sacrifice on each sampling date and the participation of our field team only consisted of receiving the tube with the collected blood and annotating the identification data of the sample, without any other participation in MACC procedures. Since our team members did not have direct participation or responsibility for the ethical aspects or humane handling of the dogs subject to the study during the sampling sessions, the evaluation or approval of the project protocols before the corresponding Ethics and Evaluation Committee of the Institute for Research of Veterinary Sciences (IRVS) of the Autonomous University of Baja California (AUBC) was not requested.

### Serum samples from stray dogs

A total of 331 whole blood samples were collected from dogs captured by MACC during the years 2017 (n = 110), 2018 (n = 88), 2019 (n = 52), and 2020 (n = 81). All laboratory procedures were performed at the Serology Laboratory (SL) of the IRVS of the AUBC, Mexico. Blood samples were collected at MACC premises. Briefly, MACC personnel sedated the dogs with xylazine and once the animal had reached deep sedation a 3 mL blood sample was collected by jugular vein puncture using evacuated tubes and passed to a member of our team to proceed with blood sample identification and storage in thermal coolers with gel refrigerants. At the end of the sampling session, blood tubes were transported to the SL at IRVS and centrifuged for 10 min at 2 000/g and 5 °C in a refrigerated centrifuge. Serum collected from blood samples was stored in 1.5 mL tubes at -20 °C until serological testing.

### Recombinant LipL32 protein antigen

The design of the rLipL32 protein antigen was based on the sequence of *Leptospira interrogans* strain 200901482 LipL32 (GenBank: JN683904.1) a 474-base pair gene fragment encoding a protein of 158 amino acids (GenBank: AEZ53279.1). Expression and synthesis of rLipL32 were performed by GenScript (Piscataway, NJ, USA) using a baculovirus expression system and a batch of 3.6 mg of rLipL32 with a concentration of 0.20 mg/milliliter and purity of 80 % was produced and delivered by GenScript.

### Positive and negative serum controls

The positive and negative serum control samples were kindly provided from a previous study[20] conducted in Culiacan, Sinaloa, Mexico, where 165 serum samples from household dogs were tested to detect antibodies to leptospira by MAT. A pool of five blood serums from samples that resulted positive for *Leptospira interrogans* serovar *Canicola* with a MAT titer of 1:100 was used as a positive control, following the World Organization for Animal Health diagnosis criteria.[21] Negative controls were obtained from a pool of five blood serum samples that tested negative for

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MAT using a panel of *Leptospira* serovars *Canicola*, *Ballum*, *Hardjo*, *Pomona*, *Pyrogenes*, *Icterohaemorrhagiae*, *Bratislava*, *Wolffii*, *Australis*, *Grippotyphosa*, *Hebdomadis*, and *Shermani*.

### *Standardization of the ELISA-LipL32*

The rLipL32 protein was adapted as a solid-phase antigen to detect antibodies in an indirect ELISA system. Optimal antigen concentration, serum samples, and enzyme conjugate antibody dilutions were determined by standard checkerboard titration.[22] Briefly, rLipL32 protein was diluted in carbonate-bicarbonate buffer pH 9.6 and adsorbed at a concentration of 200, 100, 50, 25, 10, and 5 ng per well. After incubation overnight at 4 °C the plates were ready to use. Before running the tests, the plates were washed five times with phosphate-buffered saline (PBST, pH 7.5) containing 0.05 % Tween 20. Positive and negative controls were diluted 2-fold from 1:25 to 1:200 and added in quintuplicates to appropriate wells. After incubation at 37 °C for 1 hour, plates were washed as described before, and 100 µL of anti-dog IgG HRP conjugate (Sigma-Aldrich A6792) diluted 1:5 000, 1:10 000, 1:20 000 1:40 000 and 1:80 000 in PBTS were added to individual plates.

After another round of incubation and washing, 100 µL of tetramethylbenzidine (Sigma-Aldrich T2885) substrate solution was added to each well, allowing color development for 10 min at room temperature and stopping the reaction by adding 30 µL per well of 0.16 M sulfuric acid solution. Optical density (OD) values were obtained at 450 nm using an automated ELISA reader. The criteria for selection of the best performance for the ELISA-LipL32 was based on the amount of antigen per well, the dilution of serum samples, and the dilution of the enzyme conjugate that resulted in the maximum amplitude of difference between the mean OD value of the positive control serum over the mean OD value of the negative control serum, expressed as the positive:negative ratio (P:N ratio).

### *Data analysis*

A cut-off value equal to or above the mean OD at 450 nm of the MAT pooled positive control sera was established for this work. Serum samples from stray dogs with a mean OD score equal to or higher than the cut-off value was considered positive for *Leptospira* spp. Data analysis was performed using Excel and the Statistical Analysis Systems (SAS) software version 9.4. Chi-Square test ($x^2$) was used to determine differences between the prevalence rates and the years of study. Statistical analyses or hypotheses were tested with a confidence level of 95 %.

## Results

### *Standardization of the ELISA-LipL32*

The rLipL32 protein was successfully adapted as solid phase antigen for ELISA and optimized using different positive and negative control sera concentrations, the enzyme conjugate, and the color indicator substrate in a checkerboard titration format. The results indicate that the maximum OD difference between the positive and negative reference controls was achieved by immobilizing 10 ng of rLipL32 antigen

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per well, using the control sera diluted 1:50, and the enzyme conjugate diluted 1:10 000, resulting in a positive control negative control ratio (P:N ratio) of 10.04, confirming the successful standardization and optimization of the assay (Table 1).

**Table 1.** Standardization assay of ELISA-LipL32 showing rLipL32 antigen concentration

| rLipL32 | Sera | Conjugate | P:N ratio |
|---|---|---|---|
| 100 ng | 1:50 | 1:10 000 | 6.68 |
| 50 ng | 1:50 | 1:10 000 | 6.64 |
| 20 ng | 1:50 | 1:10 000 | 8.27 |
| 10 ng* | 1:50* | 1:10 000* | 10.04* |
| 5 ng | 1:50 | 1:10 000 | 8.04 |

*ELISA-LipL32 test conditions selected to test stray dog serum samples.
Dilutions of control sera, dilution of the enzyme conjugate, and P:N ratio

## *ELISA-LipL32 in stray dogs samples*

Using the standardized and optimized ELISA-LipL32 assay, the 331 serum samples from stray dogs captured by CMCA during 2017, 2018, 2019, and 2020 were tested in duplicate while the positive and negative controls were tested in triplicates. The results of the ELISA-LipL32 indicate that the positive controls developed a mean OD of 0.821, while the negative controls developed a mean OD of 0.074 for a P:N ratio of 11.09 for the group of control sera tested in each plate run along with the field samples (Table 2).

**Table 2.** Performance of MAT of the ELISA-LipL32 system with field samples

| | Positive control | Negative control | P:N ratio |
|---|---|---|---|
| Mean DO | 0.821 | 0.074 | 11.09 |
| Standard deviation | 0.0256 | 0.0089 | |

## *Estimation of the seroprevalence of leptospirosis*

The results of the ELISA-LipL32 with serum samples from stray dogs indicate that the highest OD value obtained was 1.644, while the lowest OD value was 0.066, with a mean OD of 0.821 for the 331 samples tested. To calculate the seroprevalence of leptospirosis, an OD ≥ 0.821 was established as the cut-off value to consider a sample as positive, corresponding to the mean value of the OD of the 331 samples tested. Based on these criteria 154/331 serum samples were considered positive to the ELISA-LipL32 platform for a seroprevalence of 46.52 % for the four years of the study. The overall and yearly seroprevalence are listed in Table 3.

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**Table 3.** Seroprevalence of *Leptospira* spp. in stray dogs from Mexicali

| Year | Prevalence | Cases |
|---|---|---|
| 2017 | 46.36 %b | 51/110 |
| 2018 | 57.95 %b | 51/88 |
| 2019 | 28.85 %a | 15/52 |
| 2020 | 45.68 %b | 37/81 |
| 2017-2020 | 46.52 % | 154/331 |

Different literals between the proportions are significant ($P \leq 0.05$).

## Discussion

Leptospirosis is a neglected infectious disease reported worldwide in industrialized and developing countries. Prevalence rates are highly underestimated in part, because the symptoms produced as a result of the infection with pathogenic *Leptospira* sp. are very similar to those of other infectious diseases, making diagnosis difficult, resulting in a low number of confirmed diagnoses.[23] Additionally, in many developing nations the lack, or insufficient medical and sanitary infrastructure limits the scope of government programs and campaigns oriented toward surveillance and studying the epidemiology of leptospirosis in both human and animal populations, limiting the implementation of the appropriate control measures.[24]

In this work, we report the seroprevalence of *Leptospira* spp. in stray dogs in four years using an indirect ELISA system based on detecting IgG antibodies to rLipL32 protein in serum samples. The LipL32 protein has been successfully used in various ELISA platforms for serological diagnosis in most production and companion animal species,[18] as well as in humans.[25] The rLipL32 protein was successfully adapted as a solid-phase antigen to obtain an optimized ELISA system that produced a clear differentiation between the OD of the positive and negative controls, resulting in a robust serological platform achieving a P:N ratio of 10.04, indicating that the ELISA-LipL32 is highly sensitive as well as highly specific. Our P:N ratio results are also considerably higher than those reported in other studies using similar ELISA systems adapted with antigens obtained by chemical extraction[26] or recombinant protein antigens,[10] reporting P:N ratios $< 4.0$ on their standardization experiments.

The high number of serum samples (154/331) that developed an OD above the cut-off value (OD $\geq$ 0.821) suggests a wide distribution of *Leptospira* spp. in the stray dog population of Mexicali between 2017 and 2020. Leptospirosis in dogs is characterized by long-term leptospiruria and the stray dog population could be a significant reservoir for the spread and infection of leptospira given the close relationship and frequent contact with the human population,[7] representing an immediate health problem for the population of house dogs and other companion animals, as well as a potential public health problem for humans due to the risk of zoonotic transmission associated to this disease.

In Mexico, the information available on leptospira in dogs is very limited, mainly due to a lack of certified laboratories with diagnostic capacity and biosafety infrastructure to respond to the demand of field and clinical veterinarians for diagnostic services other than those under government programs. The overall leptospira

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prevalence of 46.52 % obtained in this study is considerably higher than the 9 % reported in household dogs from Culiacan, Mexico,[21] and similar to the prevalence of 45.2 % reported in free-roaming dogs from a marginated rural community of Yucatan, Mexico.[27] In other countries, prevalence reports of leptospira have been documented as low as 1.8 % in Australia[28] or China with leptospira prevalence reports of 7.3 %.[29] India reports a high prevalence rate of 71.1 %,[30] and Brazil reports a prevalence of 51.0 %.[31] All these studies, conducted in stray or free-roaming dogs, used MAT to diagnose *Leptospira*.

The cut-off value established for the ELISA-LipL32 system corresponds to the mean OD of the positive controls of all test runs. Although different authors report the application of three, four, or more times the value of the standard deviation added to the mean OD of the negative controls to establish the cut-off value for ELISA tests,[26, 32] when we apply those criteria to our data considering the mean OD for the negative controls (0.074) and adding five times its standard deviation (0.0116) the result is a cut-off value ≥ 0.132 where the entire 100 % of the samples analyzed would be classified as positive, probably overestimating the number of positive reactors to the ELISA-LipL32. Even if we selected a cut-off value of 50 % of the mean OD for the positive controls (≥ 0.411), the overall prevalence would be 95.77 % based on the 331 samples tested.

The serological evidence obtained in this work suggests a wide distribution of *Leptospira* spp. among the population of stray dogs in the municipality of Mexicali with high and persistent levels of infection over time (2017 to 2020). Under this scenario, it is necessary to further control the stray dog population by reducing reproduction rates and strengthening spaying and neutering programs involving health authorities, veterinary clinicians, and voluntary veterinary students along with initiatives for education to the community to promote responsible pet ownership and safe interactions with free-roaming dogs[33] to address the problem of leptospirosis comprehensively in accordance with the social and ecological characteristics of the region.

Further research is necessary to establish the situation of leptospirosis in household dogs, other companion animal species, and food animals as possible reservoirs of the disease. These studies should also assess the serological response after vaccination against leptospira and consider the ecological and epidemiological interactions of the human population under rural and urban scenarios where leptospira might be present identifying risk factors and proposing appropriate sanitary interventions for its control. To our knowledge, this is the first report of serological evidence for the presence of pathogenic strains of leptospira for the stray dog population in Mexicali, highlighting the need for further research on this important zoonotic disease in the Northwest region of Mexico.

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## Data availability

The original datasets used in this research and if applicable, supporting information files, are deposited and available for download at the SciELO Dataverse repository doi:10.48331/scielodata.UWHJM3.

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## Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

## Author contributions

Conceptualization: SM Gaxiola-Camacho, FJ Monge-Navarro.
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