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Molecular evidence for the presence of *Leptospira borgpetersenii* in synanthropic rodents in the Nautla region, Veracruz, México

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The genus *Leptospira* encompasses ten species of spirochetes capable of infecting mammals, particularly rodents. In México, studies focused on the detection of *Leptospira* sp. in rodents are scarce, all of them restricted to three states of the Gulf of México. For this reason, this work aimed to identify the diversity of *Leptospira* species associated with synanthropic rodents in Veracruz, a state where leptospirosis is endemic. Rodents were sampled with Sherman traps placed in 10 Production Units across the Nautla region. Animals were euthanized and their kidneys removed. Subsequently, a 474-bp segment of the outer membrane protein LipL32, present in all pathogenic species, was amplified and sequenced. Sequences were compared vs. reference using the BLAST algorithm: a phylogenetic reconstruction was carried out using the Maximum Likelihood method. In addition, the prevalence of infection in each Production Unit was estimated. Twenty eight rodents of a single species (*Mus musculus*) were caught. *Leptospira* DNA was detected in 17 samples (62.9 %, $CI_{95\,\%}$ 42.3 to 80.6) from seven localities in the Nautla region. The sequences recovered exhibited 99-100% identity to each other and 99 % identity with *Leptospira borgspetersenii* sequences deposited in GenBank. This study confirms the presence of *L. borgspetersenii* in rodents, particularly in *M. musculus*, in México. This study increases the inventory of pathogenic leptospires for the state of Veracruz to three species.

El género *Leptospira* engloba 10 especies de espiroquetas capaces de infectar mamíferos, particularmente roedores. En México se han realizado escasos estudios para la detección de *Leptospira* sp. en roedores, todos ellos restringidos a tres estados del Golfo de México. Por tal motivo el objetivo del presente trabajo fue identificar la diversidad de leptospiras en roedores sinantrópicas de Veracruz, un estado endémico de leptospirosis. Para la colecta de roedores, se colocaron trampas tipo Sherman en 10 unidades de producción de la región Nautla. Los animales se sacrificaron y se obtuvieron los riñones. Posteriormente se amplificó y secuenció un segmento de 474 pb de la proteína exterior de membrana *LipL32* presente en las leptospiras patógenas. Posteriormente se compararon las secuencias con las de referencia mediante el uso del algoritmo BLAST y se realizó una reconstrucción filogenética mediante el método de Máxima Verosimilitud. Adicionalmente se obtuvieron las prevalencias de la infección por unidad de producción. Se colectaron 28 roedores de una única especie (*Mus musculus*). Se detectó la presencia de ADN de *Leptospira* en 17 muestras (62.9 %; $IC_{95\,\%}$ 42.3 a 80.6) procedentes de siete localidades de la región Nautla. Las secuencias recuperadas exhibieron una similitud del 99-100 % entre si y una identidad del 99 % con secuencias de referencia de *Leptospira borgspetersenii* depositadas en GenBank. Este estudio confirma la presencia de *L. borgspetersenii* en roedores y en particular con *M. musculus* en México. Este estudio incrementa a tres especies el inventario de leptospiras patógenas para el estado de Veracruz.

Keywords: México; pathogens; small mammals; spirochetes.

Introduction

The genus *Leptospira* encompasses a set of thin bacteria with hook-shaped tips that inhabit lentic habitats worldwide, particularly wetlands (Levett 2015). Previously, the existence of two species was recorded: a saprophytic species thriving in aquatic environments (*L. biflexa*) and a pathogenic one causing a zoonosis called leptospirosis (*L. interrogans;* Levett 2015). Today, 35 species are acknowledged, classified into three groups based on their ability to infect vertebrate hosts: non-pathogenic (*e. g., L. biflexa*, *l. idonoii,* and *L. meyeri*), facultative pathogenic *(e. g., L. broomii*, *L. fainei,* and *L. wolffii*) and pathogenic (*e. g., L. alexanderi*, *L. borgpetersenii*, *L. interrogans*, and *L. kirschneri*; Bourhy *et al.* 2014; Thibeaux *et al.* 2018).

Ten pathogenic species are currently known, which have been reported in more than 160 species of wild and domestic mammals around the world (Ballados-González *et al.* 2018). In particular, rodents play a role as reservoirs; many murine species exhibit chronic infections and continually release bacteria in urine, a phenomenon called leptospiruria. Members of the family Muridae serve as the main reservoirs of *Leptospira* sp., and are deemed responsible for spreading the infection to livestock, pets, and humans (da Silva *et al.* 2010; Colombo *et al.* 2018).

In México, few studies have been conducted to detect *Leptospira* sp. in rodents, mostly in the states of Tamaulipas, Campeche, and Yucatán (Méndez *et al.* 2013; Espinosa-Martínez *et al.* 2015; Torres-Castro *et al.* 2016; Panti-May *et*

al. 2017; Torres-Castro *et al.* 2018). These studies provide molecular evidence of the presence of *L. interrogans* and *L. kirschneri* in various species of rodents of the families Cricetidae, Heteromyidae, and Muridae. However, there are other states of México where human and bovine leptospirosis is a major public health issue. Specifically, the state of Veracruz records an incidence of human leptospirosis of five cases per 100,000 inhabitants, and seroprevalence in livestock ranging between 60 % and 80 % (Moles-Cervantes *et al.* 2002; Álvarez *et al.* 2005; Sánchez-Montes *et al.* 2015; Zárate-Martínez *et al.* 2015). However, there is no evidence on the *Leptospira* species maintained in rodent populations in the region. The aim of the present work was to determine the presence and diversity of *Leptospira* sp. in synanthropic rodents in the Nautla region, Veracruz, México.

Materials and Methods

This study was conducted in well-defined and delimited areas that have facilities, machinery, and equipment to carry out livestock activities, named Livestock Production Units (LPU), in the municipalities of San Rafael, Nautla, Martínez de la Torre, Vega de Alatorre, and Misantla, in the Nautla region, Veracruz (Figure 1). This region is located in the center-northern portion of the State of Veracruz. It is bordered by the Totonac region to the north, the Capital and Mountain regions to the south, the Gulf of México to the east, and the state of Puebla to the west. This region covers an area of 3,329 km^2, with 86.1 % dedicated to farming activities; in turn, 43.2 % is covered by pastures for cattle raising.

Rodents were sampled from November 2016 to May 2017 on Production Units across the Nautla region, Veracruz. In each Production Unit, Sherman® traps were placed (8×9×23 cm), using an oat-vanilla mixture as bait attractant. Forty traps were placed per Production Unit, strategically distributed in areas with high probability of occurrence of rodents such as warehouses, farmyards, or inside households. Traps were placed in the afternoon and reviewed the next morning (before 7 am) during two trapping nights

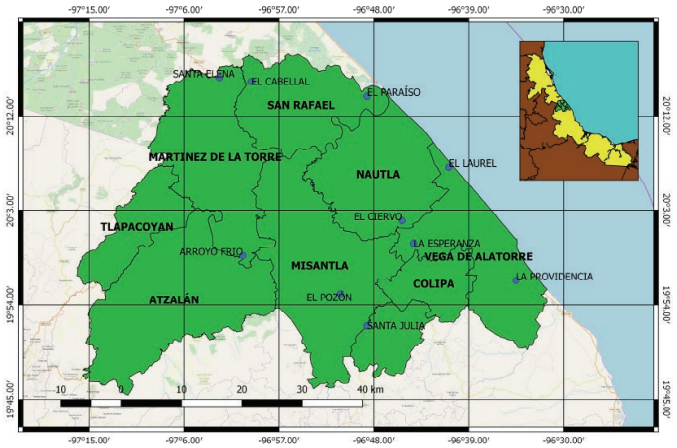

Figure 1. Map of the location of the Production Units sampled in the Nautla region, Veracruz, Mexico. The municipalities that make up the Nautla region are highlighted in green. The localities sampled are marked with blue circles.

in each locality. The rodents captured were removed from traps, identified, and processed for kidney sample collection following biosafety standards, under collection license FAUT-0250 granted by the Secretariat of Environment and Natural Resources (Semarnat). Animals were euthanized according to the protocol established by NOM-033-SAG/ZOO-2014 using ketamine (Wildlife Pharmaceutics México SA de CV 04930, México) as anesthetic agent, followed by cervical dislocation. Each rodent was placed in supine position and the absence of reflexes (corneal and podal) was confirmed before dissection to remove the kidneys. Kidney samples were placed in containers with 70 % alcohol and kept at 4 °C until processing.

DNA extraction was carried out in each sample separately, using 500 µl of a 10 % solution of the resin Chelex 100 added with 20 µl of proteinase K; then samples were incubated at 56 °C for two hours (Ballados-González *et al.* 2018). Afterward, samples were centrifuged at 15,000 rpm for 15 minutes; the supernatant was transferred to new tubes and stored at -20 °C.

Once the sample was obtained, a 474-bp segment of the outer membrane protein *LipL32*, present in the genome of the pathogenic *Leptospira* species, was amplified using the oligonucleotides (ATCTCCGTTGCACTCTTTGC) and *LipL*32 reverse (GTCCGCCTACACACCCTTTAC; Vital-Brazil *et al.* 2010). The reaction mixture consisted of 12.5 µl of a 2X solution of GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 1 µl of each oligonucleotide (2µM each), 6.5 µL of DNase-free water, and 4 µl of DNA (200-300 ng) to make a final volume of 25 µL (Espinosa-Martínez *et al.* 2015; Ballados-González *et al.* 2018). Amplicons were visualized on agarose gels using 2 % TAE buffer at 85 V for 45 min.

Positive PCR products were sent to the Biology Institute at Universidad Nacional Autónoma de México for sequencing. The resulting sequences were compared with those deposited in GenBank to determine the similarity between them using the BLAST tool.

Global alignments were carried out between the sequences produced in this study and some representative pathogenic leptospires deposited in GenBank, with the algorithm Clustal W using the software MEGA 6.0 (Tamura *et al.* 2013). The Tamura's three-parameter model of nucleotide substitution (T92) was selected based on the lowest Bayesian Information Criterion (BIC) score (2,497,948). In addition, a phylogenetic reconstruction was conducted using the maximum likelihood (ML) approach; the support of the topology was validated with 10,000 bootstrap replicates, also in MEGA 6.0.

Results

A total of 28 specimens of *Mus musculus* were collected. None of the collected animals showed signs of disease at the time of collection, nor gross evidence of renal impairment. *Leptospira* DNA was detected in 17 of the 28 samples analyzed (62.9 %; $CI_{95\%}$ 42.3 to 80.59). Positive samples

were obtained from animals collected in the localities of La Providencia, La Esperanza, Santa Julia, El Pozón, El Laurel, El Cabellal, and Tres Marías (Table 1).

The localities with the highest number of positive mice were Santa Julia (municipality of Misantla), and El Cabellal (municipality of San Rafael), with four positive specimens each (Table 1). Of the 17 positive PCR products, 12 sequences of 460 to 470 base pairs were recovered. The recovered sequences exhibited 99 % identity between them (457/460 bp), and 99 % identity (458/460 bp) with sequences of *L. borgpetersenii* deposited in GenBank. In addition, the phylogenetic analysis encompassed the sequences observed in this study along with the reference sequence of *L. borgpetersenii* in a monophyletic group with a support value of 100 (Figure 2). The sequences generated in this study were deposited in GenBank with access numbers MK568973-MK568984.

Discussion

This work is the first approximation to the study of *Leptospira* in rodents in the northern part of the state of Veracruz. Besides, it represents the first molecular confirmation of the presence of *L. borgspetersenii* in rodents, particularly in *Mus musculus*, in México (Espinosa-Martínez *et al.* 2015; Torres-Castro *et al.* 2016, 2018; Panti-May *et al.* 2017). The reference serovar of *L. borgspetersenii* isolated from *M. musculus* is Ballum, which was detected in the past century in Europe (Yager *et al.* 1953). Since then, multiple serological and molecular studies have shown that this serovar is widely distributed across *M. musculus* populations worldwide (da Silva *et al.* 2010; Matsui *et al.* 2015; Colombo *et al.* 2018). In México, studies conducted in the state of Durango have reported titers of antibodies to the *L. borgspetersenii* Ballum serovar in domestic animals such as pigs and donkeys (Alvarado-Esquivel *et al.* 2018; Cruz-Romero *et al.* 2018). Thus, it is reasonable to assume that domestic animals can be exposed to bacteria through rodents and food or water sources contaminated with rodent urine in farmyards or livestock handling and processing areas.

Table 1. Location of the Production Units sampled in the Nautla region, Veracruz, Mexico. RC = Rodents collected; PR = Positive rodents; % = Prevalence

Locality	Municipality	Latitude	Longitude	RC	PR	%
La Providencia	Vega de Alatorre	19° 56′ 0.47″	-96° 32′ 59.02″	2	1	50
El Ciervo	Vega de Alatorre	20° 02′ 00.31″	-96° 45′ 18.97″	1	0	0
La Esperanza	Vega de Alatorre	19° 59′ 53.26″	-96° 44′ 13.48″	2	1	50
Santa Julia	Misantla	19° 52′ 00.26″	-96° 48′ 38.30″	5	4	80
El Pozón	Misantla	19° 55′ 03.14″	-96° 51′ 09.71″	5	2	40
El Laurel	Vega de Alatorre	20° 07′ 04.62″	-96° 40′ 53.18″	1	1	100
El Paraíso	San Rafael	20° 13′ 51.00″	-96° 48′ 39.09″	3	0	0
El Cabellal	San Rafael	20° 15′ 15.24″	-96° 59′ 36.23″	4	4	100
Santa Elena	Martínez de la Torre	20° 13′ 55.49″	-97° 01′ 23.89″	4	3	75
Arroyo Frio	Martínez de la Torre	19° 58′ 43.10″	-97° 00′ 25.23″	1	1	100
Total				28	17	60.7

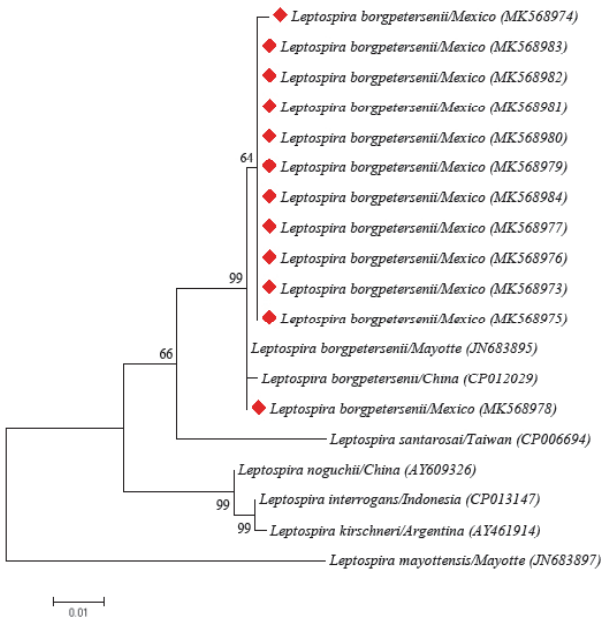

Figure 2. Phylogenetic tree of some pathogenic *Leptospira* species, obtained using the maximum likelihood (ML) approach and the Tamura's three-parameter model (TN93). The sequences recovered from rodents (*Mus musculus*) sampled in this study are marked with red diamonds.

The presence of two additional species of pathogenic leptospires (*L. weilli* and *L. noguchii*) has been confirmed in the state of Veracruz, both in kidney tissue samples of the hematophagous bat *Desmodus rotundus* and the frugivorous bat *Artibeus jamaicensis* (Ballados-González *et al.* 2018). Therefore, this study increases the inventory of pathogenic leptospires of Veracruz to three species. These findings in rodents suggest the existence of multiple transmission cycles in both wild and anthropic environments, which should be evaluated carefully.

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