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Original

Pathogenic *Leptospira* in bats from Campeche and Yucatán, Mexico

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ABSTRACT

Objective. To report the infection with *Leptospira* in the kidneys of bats from Campeche and Yucatán, Mexico, through the amplification by PCR of two different *16S RNA ribosomal* gene fragments. **Materials and methods.** Bat captures were carried out at one site in Campeche and two sites in Yucatán. Euthanasia was applied to the captured bats and a necropsy was performed to collect a renal tissue sample that was used in the total DNA extraction. Two different conventional PCR were performed for the amplification of the *16S RNA ribosomal* gene fragments. Some sequences from positive products were obtained and analyzed with bioinformatics tools to identify the infectious species of *Leptospira*. **Results.** Sixty-nine bats belonging to four families and eight different species were captured. The family with the greatest diversity was Phyllostomidae, with five species. The most captured species was *Artibeus jamaicensis* (41, 59.4%). Both PCR showed a global infection frequency of 21.7%. The infected species were *A. jamaicensis*, *Pteronotus parnellii* and *Chiroderma villosum*. The bioinformatic analysis of the positive products yielded a 99.0% identity for *Leptospira noguchii*, *Leptospira borgpetersenii*, and *Leptospira santarosai*. **Conclusions.** Some bat species of Yucatán and Campeche, Mexico, are renal carriers of pathogenic *Leptospira*, therefore participating in the transmission cycle in the region. The frequency of infection found in the renal tissue of the captured bats is higher than the one obtained from other reservoirs captured in Yucatán and Campeche. New species of bats are reported as renal *Leptospira* carriers in Mexico.

Keywords: Bacteria, chiropter, epidemiology, mammals, microbiology, spirochetes *(Source: DeSC, CAB).*

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RESUMEN

Objetivo. Reportar la infección con *Leptospira* en riñones de murciélagos de Campeche y Yucatán, México, a través de la amplificación por PCR de dos fragmentos distintos del gen *16S RNA ribosomal*. **Materiales y métodos.** Se realizaron capturas en un sitio de Campeche y dos de Yucatán. A los murciélagos capturados se les aplicó la eutanasia y se les realizó una necropsia para recolectar tejido renal que se usó en la extracción de ADN total. Se realizaron dos PCR convencionales para la amplificación de los fragmentos de *16S RNA ribosomal*. Se obtuvieron las secuencias de algunos productos positivos y se analizaron con herramientas bioinformáticas para identificar la especie infectante de *Leptospira*. **Resultados.** Se capturaron 69 murciélagos pertenecientes a cuatro familias y a ocho especies distintas. La familia con mayor diversidad fue Phyllostomidae con cinco especies. La especie con mayor frecuencia de captura fue *Artibeus jamaicensis* (41, 59.4%). Las PCR arrojaron una frecuencia de infección global de 21.7%. Las especies infectadas fueron *A. jamaicensis*, *Pteronotus parnellii* y *Chiroderma villosum*. El análisis bioinformático arrojó un 99.0% de identidad para *Leptospira noguchii*, *Leptospira borgpetersenii* y *Leptospira santarosai*. **Conclusiones.** Algunas especies de murciélagos de Yucatán y Campeche son portadores renales de leptospiras patógenas, por lo que podrían participar en el ciclo silvestre de transmisión en la región. La frecuencia de infección encontrada en los riñones de los murciélagos utilizados es mayor en comparación con aquellas obtenidas en otros reservorios de Yucatán y Campeche. Nuevas especies de murciélagos son reportadas como portadores de *Leptospira* para México.

Palabras clave: Bacteria, epidemiología, espiroquetas, mamíferos, microbiología, quiróptero *(Fuentes: DeSC, CAB).*

INTRODUCTION

Leptospira is a genus of spirochete bacteria, belonging to the order Spirochaetales and the family Leptospiraceae. According to the most recent phylogeny, these bacteria are divided into four subclades (P1, P2, S1, and S2) with different numbers of species, of which pathogens (subclass P1) cause leptospirosis in humans and animals (1). Although leptospirosis is considered a public health problem in developing countries' populations, many cases are not diagnosed because the symptoms are not specific, and the infection is usually self-limiting or has a subacute course. However, some cases can be fatal due to pulmonary or renal complications (2,3).

This disease is an endemic zoonosis in tropical and subtropical areas of Mexico. Its incidence and prevalence vary from region to region according to socioeconomic characteristics, such as hygiene and sanitary conditions, and ecological conditions such as humidity, temperature, rainfall, and floods, which contribute to the dispersion and survival of *Leptospira* in the environment. In the states of Yucatán and Campeche, located in southeastern Mexico, various prevalence or seroprevalence rates have been reported due to the strong influence of the aforementioned socioeconomic and ecological characteristics (2,3).

Outbreaks of human leptospirosis are mainly associated with the presence of reservoir animals or accidental hosts that excrete viable leptospires in their urine (because bacteria colonize and reproduce in their kidney tissue), which pollutes the environment and natural (i.e. lakes, lagoons, cenotes, rivers, etc.) or artificial water bodies (i.e. cisterns, pools, dams, tanks, etc.). Rodents *Mus musculus*, *Rattus rattus*, and *R. norvegicus* are the most important reservoirs and have therefore been identified as the most relevant route of transmission in the human populations of some tropical regions (3,4).

There is evidence that bats are reservoirs of numerous viruses that have caused epidemics in humans and animals (5,6). Recently, these animals have also been involved in the zoonotic cycle of *Leptospira* by polluting the environment or artificial bodies of water with their urine. However, the few hypotheses about the transmission mechanisms of *Leptospira* excreted by them to human populations or other animals, are still called into question (7,8).

The increase in bat groups in areas inhabited by humans is due to severe anthropogenic alterations in their natural habitat or resting sites, which expedite their migration (7). These modifications, associated with the lack of natural predators, contribute to some generalist

bats inhabiting and colonizing abandoned houses or factories, generating greater direct or indirect contact (through excreta) between them and humans or other animals (9). This aspect also increases the probability of accidental transmission of different pathogenic microorganisms such as *Leptospira*. Because of this, records of infection with pathogenic *Leptospira* species in bats are increasing around the world (8). However, in Mexico, there has only been one report, made by Ballados-González et al. (10) in the state of Veracruz (east-central Mexico), in which three species positive for infection were found: *Artibeus lituratus*, *Choeroniscus godmani***,** and *Desmodus rotundus*.

Knowing the species of pathogenic leptospires that infect bat populations, is the first step in assessing the risk of transmission to humans and other animals (11). In this context, the objective of this study was to report the infection with *Leptospira* in the kidneys of bats from Campeche and Yucatán, Mexico, through the amplification of two different fragments of the 16S RNA ribosomal gene (16S-rRNA).

MATERIALS AND METHODS

Ethical guidelines. The extraction of bats was approved by the Ministry of Environment and Natural Resources of Mexico (minutes: SGPA/DGVS/03705/17; SGPA/DGVS/01186/17). The capture, sacrifice, and collection of biological samples in the studied bats were approved by the Bioethics Committee of the Faculty of Veterinary Medicine and Zootechnics (*FMVZ* for its acronym in Spanish) of the Autonomous University of Yucatan (*UADY* for its acronym in Spanish) (minutes: CB-CCBA-I-2018-001).

The capture of bats and study sites. The captures were made as part of a project whose objective was the identification of wild mammal species which are potential reservoirs of the Ebola virus in south-eastern Mexico. Therefore, it was decided to work in sites with easy access and which had the minimum facilities and infrastructure necessary to set up a laboratory in which bats and other captured animals (not included in this research) were processed.

The captures were made in May, August and September 2017 in three sites. The first capture was carried out at the Center for Studies for Wildlife Conservation and Research (*CECIVS* for its acronym in Spanish) (19°56'-19°57' N and 90°22'-90°22' W), located in the municipality of Hampolol in the state of Campeche. This site has a sub-humid warm climate, an average annual temperature of 26.1°C and an annual rainfall of over 1027 mm. The forest area is made up of different types of tropical forests, such as medium sub-deciduous, medium sub-evergreen and low flood sub-evergreen, and aquatic and secondary vegetation. This vegetation has undergone a certain degree of alteration, as this site was part of a pre-Hispanic settlement and later a farm (12).

The second capture was carried out on a livestock ranch (21°24'-21°23' N and 88°30'-88°19' W) located in the municipality of Panabá in the state of Yucatán. This area has a sub-humid warm climate with rains in summer, an average annual temperature of 24 to 26°C and a rainfall during the winter that can vary from 600 to 1,500 mm. The vegetation is mainly deciduous rainforest. However, due to human activities in the area (mainly agricultural and livestock), it has been severely transformed (13).

The third capture was carried out in the *FMVZ-UADY* (20°49'-20°51' N and 89°38'-89°39' W), located in the municipality of Mérida, the capital of Yucatán. It has a sub-humid warm climate with rains in summer, an average annual temperature exceeding 26°C and an annual rainfall between 675 and 975 mm. Most of the surface is covered with secondary vegetation in different stages of regeneration over a period of 20 to 30 years (85% of the total area); the rest (15%) is distributed throughout land with agricultural (cornfields, grasslands, henequenals or other crops) or livestock uses, plots (family gardens), or areas with some type of construction (streets and homes) (14).

Bat processing and biological sampling. Each site was sampled for up to three nights. Three mist nets (12.0 m x 2.6 m) were placed in locations near water reservoirs, fruit trees, and caves. The nets were opened for five hours (18:00 to 23:00 h), during which time increased activity was observed for different groups of bats, carrying out the reviews every 20 minutes.

The captured bats were removed from the nets using leather work gloves and placed in cloth bags to transport them to the assembled laboratory for processing within the study sites. All specimens were identified using a taxonomic key (15).

All the bats were euthanized by intraperitoneal sodium pentobarbital overdose, prior to isoflurane anaesthesia, as previously described (16). Subsequently, a necropsy was performed to collect a portion of the kidney. This was deposited in sterile 1.5ml vials (Eppendorf®, Germany) immersed in pure ethanol. The personnel involved in the processing of the animals used double latex gloves, specialized masks, disposable gowns, closed shoes, etc., to prevent contact with the urine, feces, saliva, or blood of the bats.

The kidney portions were preserved at 4°C during their transfer to the Emerging and Reemerging Diseases Laboratory (*LEER* by its acronym in Spanish) of the Research Center "Dr. Hideyo Noguchi" (CIR by its acronym in Spanish) of the UADY, where they were stored at -80°C until used in total DNA extraction.

Total DNA extraction and identification of *Leptospira*. All tissues were washed with double-distilled water for approximately five minutes to remove excess alcohol and have as little effect as possible on the quality of the extracted DNA.

The total DNA extraction from the renal tissue was performed as described in the DNeasy® Blood & Tissue extraction kit (QIAGEN®, Germany), after digestion with proteinase K (Omega Bio-tek® Inc., United States of America [USA]). Subsequently, it was quantified and evaluated on a spectrophotometer (NanoDrop 2000™, Thermo Scientific®, USA), and finally stored at -70°C until its use in molecular detection.

To identify the infection with *Leptospira*, two conventional polymerase chain reactions (PCR) were performed to obtain the amplification of two distinct fragments of the *16S-rRNA* gene.

In the first, oligonucleotides 16S3 (5'-ATCCTCATGGCCTTTATGTC-3') (forward) and 16SR (5'-GTCCGCCTACACACCCTTTAC-3') (reverse) as previously described (17) were used, which amplify a segment of 150 base pairs (bp). The final concentrations of the reagents, in a final volume of 25 µl, were: 1X PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM each oligonucleotide, 1U Taq polymerase (Thermo Scientific®, USA), and the volume was increased to 25 µl with molecular biology grade water. The conditions used in the thermal cycler were: initial denaturation at 95°C for five minutes, followed by 34 cycles with stages of 95°C for 30 seconds, 49°C for 30 seconds and 72°C for 30 seconds. The final extension was at 72°C for five minutes.

The second PCR was only performed on the positive extractions to the previous reaction. The oligonucleotides used were 16S4 (5'-AGTGAACGGGATTAGATACC-3') (forward) and 16S6 (5'-CCTAGACATAAAGGCCATGA-3') (reverse) (18), which amplify a segment (different from the first reaction) of 440 bp of the afore-mentioned gene. The volume and final concentrations of each reagent were equal to those of the first PCR (150 bp). The conditions of the thermal cycler were: initial denaturation at 95°C for five minutes, followed by 34 cycles with stages of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. The final extension was at 72°C for five minutes.

In all reactions, a positive control (*Leptospira* DNA typified as *L. interrogans*) and negative control (sterile water) were included. Electrophoresis was performed on agarose gels (1%) stained with ethidium bromide. The results were recorded with a photo-documentation system (Biorad®, USA).

Bioinformatic Analysis. Due to limitations on the economic resources, only some products positive to the second reaction (440 bp) were purified using the Gel DNA Recovery kit (Zymoclean®, USA), following the instructions established by the commercial house, and sent for sequencing by Sanger method to the private laboratory DYMIGEN® (Mérida, Mexico). The products sent were selected according to their concentration and purity, obtained by NanoDrop2000™ (Thermo Scientific®, USA).

With the received sequences (in forward and reverse directions), a consensus sequence was generated (eliminating fragments corresponding to oligonucleotides 16S4 and 16S6) with the MEGA V7.0® software, and examined with the Basic Local Alignment Search (BLAST) tool, using the Megablast® algorithm, both developed by the National Institute of Health (NIH), with the objective of knowing the identity and coverage of *Leptospira* species in the infected bats.

RESULTS

With a sampling effort of 59 hours/net, a total of 69 bats were captured from four families: Noctilionidae, Mormoopidae, Phyllostomidae, and Vespertilionidae, and eight species: *Noctilio leporinus*, *Pteronotus parnellii*, *Glossophaga soricina*, *Carollia sowelli*, *Artibeus jamaicensis*, *A. lituratus*, *Chiroderma villosum*, and *Rhogeessa aeneus*. Five captured species belonged to the family Phyllostomidae. *Artibeus jamaicensis* was the species with the highest percentage of captured specimens (59.4%, 41/69). The site with the highest percentage of captures was the *CECIVS* (36.2%, 25/69), it was also the one with the highest number of species and families captured (Table 1). Regarding eating habits, fishing, frugivorous, frugivorous/nectarivorous, and insectivorous species were captured.

The PCR for the detection of the *16S-rRNA* gene showed a infection global frequency of 21.7% (15/69). Of the infected specimens, 11 were *A. jamaicensis* (73.4%), 2 *P. parnellii* (13.3%), and 2 *C. villosum* (13.3%). At least one infected individual was identified at each study site, being the *FMVZ* in which the highest number was obtained with eight (53.3%, 8/15), all belonging to the *A. jamaicensis* species. Infected specimens of *P. parnellii* and *C. villosum* were captured in *CECIVS* and Panabá, respectively.

Information corresponding only to the bats positive for infection is presented in Table 2.

Table 2. Study site, species, the total number of individuals captured by species, and the number of infected individuals by species of the bats studied of Campeche and Yucatán, Mexico.

Study site	Species	#ICS	#IIS
CECIVS	*Pteronotus parnellii*	6	2 (33.3)
	Artibeus jamaicensis	7	2 (28.6)
Panabá	*Chiroderma villosum*	5	2 (40.0)
	Artibeus jamaicensis	12	1 (8.33)
FMVZ	*Artibeus jamaicensis*	22	8 (36.4)

#ICS=Total number of individuals captured by species.
#IIS= Number of infected individuals by species (%)

The bioinformatic analysis of the edited sequences showed 99.0% identity and coverage for the pathogenic species *Leptospira noguchii*, *L. borgpetersenii*, and *L. santarosai* for the positive products of the *P. parnellii* and *C. villosum* bats, with *L. borgpetersenii* being the only one species identified in both (Table 3). The number of bp that were used at the time of providing these identity values was 440.

Table 1. Family, species, and the number of bats captured for each study site in Campeche (*CECIVS*) and Yucatán (Panabá and *FMVZ*).

Family	Species	Individuals captured for each study site			Total number of individuals captured (%)
		Campeche	Yucatán		
		CECIVS	**Panabá**	***FMVZ***	
Noctilionidae	*Noctilio leporinus*	6	0	0	6 (8.7)
Mormoopidae	*Pteronotus parnellii*	6	0	0	6 (8.7)
Phyllostomidae	*Glossophaga soricina*	1	3	0	4 (5.8)
	Carollia sowelli	2	0	0	2 (2.9)
	Artibeus lituratus	1	1	0	2 (2.9)
	Artibeus jamaicensis	7	12	22	41 (59.4)
	Chiroderma villosum	1	5	0	6 (8.7)
Vespertilionidae	*Rhogeessa aeneus*	1	1	0	2 (2.9)
Total (%):		25 (36.2)	22 (31.9)	22 (31.9)	69 (100)

Table 3. Bat species, study site, *Leptospira* species, GenBank accession number of the homologous sequence, and BLAST analysis results of the PCR products positive for the *Leptospira 16S-rRNA* gene.

Bat species	Study site	*Leptospira* species	#GA	BIC
Pteronotus parnellii	*CECIVS*	*L. santarosai*	CP028377.1	99.0
		L. borgpetersenii	MH059524.1	99.0
Chiroderma villosum	Panabá	*L. borgpetersenii*	AY995713.1	99.0
		L. noguchii	EU349495.1	99.0

#GA=GenBank accession number of the homologous sequence. BIC=BLAST analysis results (%); identity and coverage.

DISCUSSION

Bats from the Yucatan Peninsula have been identified as reservoirs of different viruses of the *Flavivirus* genus (6) and as accidental hosts of the *Toxoplasma gondii* (16) and *Trypanosoma cruzi* (19,20) protozoa. Therefore, this study increases knowledge about the participation of these mammals in the epidemiological cycle of emerging or re-emerging infectious agents indicated in the region with relevance to public health and animal health, as is *Leptospira* (21).

In Yucatan, infection with pathogenic leptospires has been described in rodent kidneys captured in peridomestic and wild environments (17,22,23,24); while, in Campeche, the infection has been detected in wild rodents (25). Consequently, the present findings are the first in bats from Yucatán and Campeche, Mexico.

The establishment and colonization of *Leptospira* in the renal tissue of animal reservoirs have been related to the increase of new cases and the generation of leptospirosis outbreaks in humans or domestic animals (susceptible hosts) of some tropical endemic regions. This is due to the excretion and dispersion of bacteria in their urine and the subsequent environmental or water pollution where bacteria survive and remain infective for varying periods of time (3,4,26).

Interestingly, the frequency of infection found in the renal tissue of the bats used in this work (21.7%) is higher compared to that obtained in renal tissue from other reservoirs captured in Yucatán, such as mice (1.5%, 2/130) (17), rats (1.4%, 1/73) (23) and wild rodents (4.5%, 1/22) (24), and in wild rodents captured in Campeche (20%, 2/10) (25). This is possibly related to greater and more frequent contact between bats and leptospires that remain infective in the humid soils in which these animals usually live, rest, or transit (27). However, more epidemiological investigations are necessary to confirm this hypothesis.

On the other hand, this same frequency of infection is lower compared to that previously reported by Ballados-González et al (10) in bats from Veracruz, Mexico (30.9%, 25/81). It is also lower compared to studies conducted in bats from other countries on the American continent, such as Colombia (26.9%, 7/26) (28) and Brazil (39.1%, 36/92) (29), but it is higher compared to research in bats from Argentina (2%, 14/70) (30), Peru (3.4%, 20 / 2,237) (31), and another study from Brazil (2%, 6/343) (32). All these findings show wide variations in the frequencies of infection with *Leptospira* in this group of mammals (28).

Although several studies have identified pathogenic leptospires in bats around the world, there is controversy about their participation in the zoonotic transmission cycle of these bacteria and the consequent presentation of the disease in humans (8,27). However, due to the severe and numerous anthropogenic changes (mainly in land use), the mobility capacity of the Chiroptera, and the high numbers of their populations, the likelihood of contact between these animals and humans has increased, together with the risk of transmission of pathogenic *Leptospira* (28,32).

Some hypotheses have been formulated on the mechanisms of transmission of *Leptospira* in bats, regardless of their feeding behavior. One of them is the contact with the urine of other carrier individuals belonging to the same colony or group, due to the marked gregarious behavior that allows bats of different species or a single species to coexist in the same habitat (8), coupled with the grooming behavior that propitiates the accidental ingestion of remains (drops) of urine present in the body or on the wings (33). In the same way, the consumption of water from natural or artificial bodies, contaminated with viable *Leptospira* can be another route of transmission, as usually happens with other types of mammals (26). This can explain the diversity of species of infected bats (which drink from the same source of contaminated water), as well as the high prevalence and different species of leptospires that infect these animals (9). Finally, indirect contact with wet soil contaminated with viable

leptospires has also been suggested as a possible transmission mechanism (29), especially in species of bats that consume insects with substrate remains in their bodies or on their extremities (34).

This study shows that, following the identification of the infection in *P. parnellii* and *C. villosum,* a greater number of bat species are carriers of *Leptospira* in Mexico. Previously, Ballados-González et al (10) reported infection in *A. lituratus*, *C. godmani* and *D. rotundus*.

Artibeus jamaicensis is the only positive species for Yucatán and Campeche that has previously been described as infected with *Leptospira,* on the island of Trinidad, for the American continent (35).

Regarding the pathogenic species of *Leptospira*, Ballados-González et al. (10) described the infection with *L. noguchii*, *L. weilii*, and *L. interrogans*. Therefore, according to these records, this research demonstrates for the first time the infection with *L. borgpetersenii* and *L. santarosai* in bats of Mexico. *Leptospira noguchii* is the only species located in bats of the three states (Veracruz, Campeche, and Yucatán). Likewise, regarding the pathogenic leptospires reported in the research carried out in bats of the American continent (28,29,30,31,32,35), this investigation unprecedently identifies the infection in *L. santarosai*, which is also probable worldwide (8,9).

Another contribution of this study is the increase in the diversity of pathogenic species of *Leptospira* (*L. borgpetersenii*, *L. santarosai*, and *L. noguchii*) in renal carriers of Yucatán and Campeche, since *L. interrogans* has previously been described in synanthropic mice (*M. musculus*) and wild rodents such as *Heteromys gaumeri* and *Ototylomys phyllotis* (17,23,24), and *L. kirschneri* in *R. rattus* (17).

Unfortunately, the positive findings in *A. jamaicensis* (which was the species with the highest number of captures and positives for the infection in the present work) could not be sent for sequencing and analysis, which means a limitation in the results, since these individuals are probably infected with other species of *Leptospira* prevalent in the region. For this reason, it is necessary to make a new capture to determine the epidemiological value of this species in the transmission cycle.

The presence of renal carrier bats of *Leptospira* may have implications for public health. Vashi et al (7) described a case of leptospirosis in an adult person, acquired by indirect contact with a bat whose urine probably contaminated an artificial body of water. Considering the above and the characteristics of *CECIVS*, such as the extensive natural bodies of water with access by the public, this route of transmission could occur in users through contact with water which has been contaminated by urine of infected bat (36). On the other hand, considering that two of the species of generalist bats (adapted to houses and other constructions) captured in Yucatán and Campeche were positive to infection with *Leptospira*, the risk of transmission to people and pets by contact with their urine may increase (30,32). In this sense, the species *A. jamaicensis* has already been identified in the surrounding areas of rural dwellings in Yucatán (19).

Conversely, the indirect transmission to humans and domestic animals of the leptospires that infect bats can occur by the participation of other reservoirs, particularly those that inhabit the same habitats and feed on the ground, such as mice (*M. musculus*) or rats (*R. rattus*) (11,31), which have been identified in the interior or nearby surroundings of rural and suburban houses in Yucatán (23).

Conflict of interests

The authors of this manuscript declare that there is no conflict of interest.

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