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SHORT COMMUNICATION

Absence of molecular evidence of *Leptospira* spp. in urine samples collected from rodents captured in Yucatán, México

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ABSTRACT. *Leptospira* spp. is a spirochete bacteria, causal agent of leptospirosis, zoonotic disease endemic in México that represents a serious public health and veterinary problem. Rodents are recognised as the most important reservoirs of this bacteria, which is transmitted mainly through direct or indirect contact with the *Leptospira* spp. excreted in the urine of infected individuals. The aim of this study was to evaluate the circulation of *Leptospira* spp. in urine samples of wild and synanthropic rodents from Yucatán, México. Eighty-four rodents were captured in the community of Cenotillo, Yucatán. Twenty-six urine samples were collected from the bladder and were used in the total DNA extraction. The identification of *Leptospira* spp. was intended through the polymerase chain reaction test in its endpoint variant. No evidence of *Leptospira* spp. was found in the urine samples. It is necessary to use other tissues for the identification of *Leptospira* spp., before concluding that the rodents used in the present study are not reservoirs of this bacteria.

Key words: urine, rodents, *Leptospira* spp., Yucatán, México.

RESUMEN. *Leptospira* spp. es una bacteria espiroqueta, agente causal de la leptospirosis, enfermedad zoonótica endémica en México que representa un serio problema de salud pública y veterinaria. Los roedores son reconocidos como los más importantes reservorios de la bacteria, la cual es transmitida principalmente por contacto directo o indirecto con *Leptospira* spp. contenidas en orina de individuos infectados. El objetivo de este estudio fue evaluar la circulación de *Leptospira* spp. en muestras de orina de roedores silvestres y sinantrópicos de Yucatán, México. Ochenta y cuatro roedores fueron capturados en la comunidad de Cenotillo, Yucatán. Se recolectaron 26 muestras de orina de la vejiga y fueron usadas en la extracción de ADN total. La identificación de *Leptospira* spp. se pretendió por medio de la prueba de reacción en cadena de la polimerasa en su variante punto final. No se encontró evidencia de *Leptospira* spp. en las muestras de orina. Es necesario emplear otros tejidos para la identificación de *Leptospira* spp. antes de concluir que los roedores usados en el presente estudio no son reservorios de esta bacteria.

Palabras clave: orina, roedores, *Leptospira* sp., Yucatán, México.

INRODUCTION

Leptospira spp. is recognised as the causal agent of leptospirosis, reemerging zoonotic disease endemic in several countries of the American continent, but with higher rates of incidence and prevalence in tropical or subtropical areas (Nájera *et al* 2005). In México, leptospirosis is a public health and veterinary problem (Torres-Castro *et al* 2016[a]), even though the disease is not notifiable and species or serovars involved in the infection are rarely reported (Sánchez-Montes *et al* 2015).

Leptospira spp. is capable to infect more than 160 intermediate hosts, causing nonspecific clinical manifestations. Currently, there are 22 species of *Leptospira* spp. divided into three clades: 1. Seven saprophytic species: *L. biflexa*, *L. wolbachii*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*, *L. yanagawae*, and *L. idonii*; 2. Ten pathogenic species: *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, *L. kmetyi*, *L. alstoni*, and *L. mayottensis*; 3. Five intermediate species: *L. inadai*, *L. broomii*, *L. fainei*, *L. wolffii*, and *L. licerasiae*; being the pathogenic species the most relevant because they cause disease (Bourhy *et al* 2014).

Rodents have been reported as the primary reservoirs of pathogenic leptospires, reason why there are numerous investigations worldwide with variable infection rates (Agudelo-Flórez *et al* 2009, Sumanta *et al* 2015). This characteristic is due to the ability of *Leptospira* spp. to develop and reproduce in kidney cells, even causing notable tissue damages (Torres-Castro *et al* 2016[b]), being excreted through the urine (leptospiruria) and contaminating the sources of water and food, main route of infection of susceptible hosts (Nájera *et al* 2005). Experimental studies have shown that a rat infected with *Leptospira* spp., is able to excrete up to 6.1×10^6 genomic equivalents, measured by quantitative polymerase chain reaction (qPCR) (Costa *et al* 2015).

In México, reports of the *Leptospira* spp. circulation in wild or synanthropic rodents are scarce. Likewise, Panti-May *et al* (2016), indicates that *Mus musculus* and *Rattus rattus* are the most abundant rodent species in rural and urban environments of Yucatán, positioning them as important sentinels of the circulation of zoonotic agents.

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Diagnosis of leptospirosis is mainly performed by microagglutination (MAT) and bacterial culture; however, both tests have several limitations, especially in the detection of chronic infections and the prolonged time because *Leptospira* spp. grows slowly (Musso and La Scola 2013). Thi is why molecular test protocols such as PCR, may facilitate early diagnosis in individuals with suggestive symptoms of leptospirosis, as well as the identification of animals as carriers through urine samples (Sedano *et al* 2016).

The aim of the present study was to evaluate the presence of *Leptospira* spp. in urine samples collected from synanthropic and wild rodents, captured in a municipality of Yucatán, México, through the use of a PCR test in its endpoint variant.

MATERIAL AND METHODS

The study was carried out in the rural community of Cenotillo, Yucatán, México (20.966°, –88.604°). The regional climate is tropical (Aw) with an average annual temperature of 26.3 °C. The annual average rainfall is 1,200 mm and usually occurs in May and July; the predominant vegetation surrounding the locality is low deciduous forest with small extensions of median forest and patches of forage grass. This study community was chosen because shares environmental and demographic characteristics to those of the study site described in Torres-Castro *et al* (2014), in which previously were captured positive rodents to *Leptospira* spp. in Yucatán.

The capture and the rodents sampling were approved by the Ethics Committee of the *Campus de Ciencias Biológicas y Agropecuarias* (CCBA) of the *Universidad Autónoma de Yucatán* (UADY) (registration number: CB-CCBA-M-2016-004) and the *Secretaría de Medio Ambiente y Recursos Naturales* from México (Registry: SGPA / DGVS / 00867/17).

Rodents were captured in 40 dwellings chosen for convenience, following the methodology described by Torres-Castro *et al* (2014), and in two small areas of forest without anthropogenic effect, located at 9 Km from the urban settlement. The capture was carried out under the statutes of the American Society of Mammalogists (ASM) (Sikes *et al* 2011). Sampling was conducted during July and August 2016. Species identification of the captured rodents was carried out by experienced veterinarians and biologists.

For rodent sampling, the urban settlement of the study site was divided into four quadrants, drawing two perpendicular axes at its center. Ten houses per quadrant were selected for convenience and were sampled for two consecutive nights during two weeks of each month. In each house, 12 Sherman traps (8cm x 9cm x 23cm; H.B. Sherman traps; Florida, USA) were placed and distributed in the dwelling and the backyard, close to signs of rodent activity and potential sources of food or harborage. For the sampling in the sylvatic areas, 100 Sherman traps were distributed through ten linear transects, placing a trap each 5-6 m. The capture was made the same days and weeks as the urban quadrants.

All traps were placed in the morning and checked the next day; those with capture were replaced by another and located in the same place. The bait used was a mixture of oat flakes and artificial vanilla essence.

All captured rodents were transferred to a room enabled in the study site. The animals were anaesthetised whit a intraperitoneal injection of sodium pentobarbital (130 mg/kg) and euthanised by cervical dislocation, according to the American Veterinary Medical Association (AVMA) (Leary *et al* 2013). After euthanasia, somatic data were determined, as well as the species, sex, and age of all individuals. A necropsy was performed to collect the urine (approximately 300 µl), which was taken directly from the bladder (when it was full) using insulin syringes (TERUMO®, Tokyo, Japan), were deposited in 1.5ml microcentrifuge tubes (Eppendorf®, Hamburg, Germany), and stored at –70 °C until use in total DNA extraction.

Before DNA extraction protocol, the urine samples were centrifuged for 15 min at 10,000 rpm at 4 °C, with the purpose to discard part of the supernatant and collect the precipitate (pellet) formed at the bottom of the microcentrifuge tube. All samples were processed with the kit QIAamp DNA Mini Kit® (QIAGEN®; Hilden, Germany), protocol DNA Purification from blood or body fluids, following the manufacturer's specifications. The extracted DNA was quantified in a spectrophotometer (NanoDrop 2000™, Thermo Scientific®, Wilmington, USA) and stored at –20 °C until used in the molecular assay.

The detection of *Leptospira* spp. was intended through two PCR endpoint assays, like a previous methodology described by Torres-Castro *et al* (2014): in the first assay, primer set 16S3 (sense) (Haake *et al* 2004) and 16SR (antisense) (Shukla *et al* 2003) were used, which amplify a segment of 150bp belonging to the 16S rRNA gene of *Leptospira* spp. Additionally, these results were corroborated with a second PCR endpoint, using the primer set 16S5 (sense) (Haake *et al* 2004) and 16SR (antisense) (Shukla *et al* 2003), which amplify a fragment of 1,005bp belonging to the same 16S rRNA gene. This gene is the most used and accepted for molecular identification of *Leptospira* spp. (Sumanta *et al* 2015).

The reagents used in both reactions had the following final concentrations: PCR Buffer 5X, 2.5mM $MgCl_2$, 0.2 mm dNTP's, 0.2 mm of each primer, 1U Taq polymerase (Thermo Scientific Inc., Massachusetts, USA), and double-distilled water for laboratory use. Three microliters of DNA extraction were used as template. The conditions in the thermal cycler for both reactions were: an initial denaturation cycle at 95 °C for five minutes, followed by 34 cycles at 94 °C for 45 seconds, 94 °C for one minute, and 72 °C for two minutes. The final extension was at 72 °C for five minutes.

Table 1. Species, sex, and age of the rodents captured in Cenotillo, Yucatán, México.

Species	Sex			Age		
	Male	Female	Total (%)	Sub-adult	Adult	Total (%)
Rattus rattus	7	18	25 (29.9)	14	11	25 (29.9)
Mus musculus	14	10	24 (28.7)	20	4	24 (28.7)
Heteromys Gaumeri	5	12	17 (20.2)	8	9	17 (20.2)
Peromyscus Yucatanicus	2	6	8 (9.5)	4	4	8 (9.5)
Ototyllomys Phyllotis	3	5	8 (9.5)	1	7	8 (9.5)
Sigmodon Hispidus	0	1	1 (1.1)	0	1	1 (1.1)
Peromyscus Leucopus	0	1	1 (1.1)	1	0	1 (1.1)
Total	31	53	84 (100)	48	36	84 (100)

All reactions included positive (DNA extracted from a culture of *Leptospira* spp., donated by the *Laboratorio de Enfermedades Infecciosas y Parasitarias-Facultad de Medicina*, *Universidad Autónoma de Yucatán*) and negative (sterile water) controls. The electrophoresis of the PCR products was performed on 1% agarose gels, stained with ethidium bromide, and visualized by photo documentation (Bio-rad®, California, USA).

RESULTS AND DISCUSSION

A total of 84 rodents belonging to seven species were captured. Table 1 summarises the number of individuals captured for each species, as well as the frequency of age and sex. Likewise, table 2 summarises the species and the number of individuals to whom urine collection was possible. No leptospiral DNA was found in both molecular reactions (figure 1).

Worldwide, few studies conducted in reservoirs have used urine as a biological sample for the detection of *Leptospira* spp., due the difficulty that represents the collection of this waste, especially in rodents captured in natural environments. Pathogenic leptospires colonize the renal tubules of reservoir or hosts and are excreted via urine into the environment. Asymptomatic reservoir or hosts include a wide range of wild and domestic animal species such as cattle, dogs, and rodents, that can persistently excrete large numbers of pathogenic leptospires over many months (Nally *et al* 2015).

In the present study, all urine samples were negative to leptospiral DNA. Esfandari *et al* (2015), reported a low frequency (0.7%; 1/150) of *Leptospira* spp. in urine from synanthropic rodents captured in ten locations of Iran, results that shows a low leptospiruria rate like this research.

The presented negative results may be a consequence of the reduced number in the rodents used. In a previous work made by Torres-Castro *et al* (2014) whit synanthropic rodents from Yucatán, the rate of *Leptospira* spp. infection determined in renal tissue (4.81%), was relatively low compared whit the total number of used individuals (187); likewise, it is probable that the rodents considered in our research were not in leptospiruria phase if not in chronic infection, stage in which the bacteria is shelter in the different organs of the affected individual and is not necessarily excreted by the urine (Costa *et al* 2015, Torres-Castro *et al* 2016[b]).

Another factor to consider in our negative results is the circulating serovar. Thiermann (1981), demonstrated that Icterohaemorrhagiae serovar persist for more time

Table 2. Species and frequencies of the individuals used in the PCR test.

Species	Frequence (%)
Heteromys gaumeri	10 (38.5)
Rattus rattus	6 (23)
Ototylomys phyllotis	5 (19.3)
Peromyscus yucatanicus	3 (11.5)
Mus musculus	2 (7.7)
Total	26 (100)

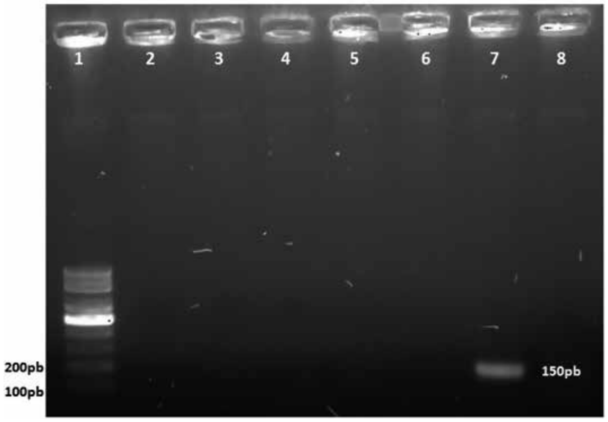

Figure 1. One percent agarose gel, stained with ethidium bromide, showing some negative PCR products to *Leptospira* spp. 1: Molecular weight marker; 2-6: Negative products; 7: Positive control; 8: Negative control.

(approximately 220 days) in renal tissue compared to Grippotyphosa serovar (approximately 40 days), so the time of leptospiruria is different between serovars. Previously, Torres-Castro *et al* (2014), identified *L. interrogans* and L. *kischneri* species in renal tissue of *M. musculus* and *R. rattus* captured in a rural community of Yucatán, México, whose serovars may have different times of excretion.

Wild or synanthropic animals are relevant in the *Leptospira* spp. infectious cycle, because these can be responsible for the circulation of several specific serovars in a determined region (Millán *et al* 2009). Although some serovars are associated with specific hosts, all animals are susceptible to infection with any serovars belonging to pathogenic and even intermediate species (Bourhy *et al* 2014). Likewise, Agudelo-Flórez *et al* (2009), reported that the distribution of leptospirosis in humans occurs mainly in areas with high population densities of rodents, as well as in areas with insufficient measures of waste collection and poor sanitary conditions.

In México, particularly in the Yucatán Peninsula, recent studies identified the native rodents *Heteromys gaumeri* and *Ototylomys phyllotis* (Espinosa-Martínez *et al* 2015) and the synanthropic rodents *M. musculus* and *R. rattus* (Torres-Castro *et al* 2014), as chronic reservoirs of pathogenic leptospires. Likewise, other wild and domestic animals (Vado-Solís *et al* 2002) have been positive to the *Leptospira* spp. circulation. These studies suggest the importance of leptospirosis in animal health in the region; however, Reyes-Novelo *et al* (2011) highlight the lack of epidemiological studies in other mammals of México, wild or synanthropic, that could act as reservoirs. On the other hand, Sánchez-Montes *et al* (2015) reported 56 cases of human leptospirosis in Yucatán between 2000-2010, numbers that could increase in the future.

Although we did not found leptospiral DNA in urine of the rodents used in our study, it is necessary to use other organs or tissues in the molecular reaction test to characterise the species circulating in the region. Also, the use of different diagnostic tests such as bacterial culture could help in the positive diagnosis. It is advisable to increase the number of captured individuals, study sites and sampling time, to improve the probability of the detection of *Leptospira* spp.

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