First molecular evidence of Leptospira spp. in synanthropic rodents captured in Yucatan, Mexico

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SUMMARY

Leptospira spp. is the causal agent of leptospirosis, an anthropozoonotic disease distributed worldwide. In Mexico, the disease is recognized as a human and livestock health problem. The synanthropic rodents *Mus musculus* and *Rattus rattus* constitute some of the most important reservoirs of the disease. The objective of this study was to use conventional PCR to investigate the condition of the agents of *Leptospira* spp. in *M. musculus* and *R. rattus* captured in a rural community in Yucatan, Mexico, and to identify the species involved in infection through sequencing and phylogenetic analysis. A total of 130 *M. musculus* and 57 *R. rattus* specimens were used. DNA was extracted from the kidney tissue and a PCR-based test was conducted, yielding a total positivity for *Leptospira* spp. of 4.81% (9/187). Sequencing and phylogenetic analysis of the PCR products identified the presence of the pathogenic species *L. interrogans* and *L. kirschneri*. This study presents the first molecular evidence of infection by *Leptospira* spp. in synanthropic rodents in Yucatan, Mexico.

Keywords : *Leptospira* spp., *Mus musculus*, *Rattus rattus*, PCR, Yucatan

RESUME

Première preuve moléculaire de *Leptospira* spp. sur des rongeurs synanthropes capturés dans le Yucatán, au Mexique

Leptospira spp. est l'agent causal de la leptospirose, une maladie anthropozoonotiques distribué dans le monde entier. Au Mexique, la maladie est reconnue comme un problème de santé humaine et animale. Les rongeurs synanthropes *Mus musculus* et *Rattus rattus* constituent certains des réservoirs les plus importants de la maladie. L'objectif de cette étude était d'utiliser la PCR classique pour déterminer la présence de *Leptospira* spp. dans des réservoirs naturels constitués par *M. musculus* et *R. rattus* capturés dans une communauté rurale dans le Yucatan, au Mexique, et d'identifier les espèces impliquées dans l'infection par le séquençage et l'analyse phylogénétique. Au total 130 spécimens de *M. musculus* et 57 de *R. rattus* ont été analysés. L'ADN a été extrait à partir du rein et un test basé sur la PCR a été effectué. Un niveau de positivité en *Leptospira* spp. de 4,81% (9/187) a été observé. Le séquençage et l'analyse phylogénétique des produits de PCR permis d'identifier la présence des espèces pathogènes *L. interrogans* et *L. kirschneri*. Cette étude présente la première preuve moléculaire de l'infection par *Leptospira* spp. chez les rongeurs synanthropes dans le Yucatan, au Mexique.

Mots-clés : *Leptospira* spp., *Mus musculus*, *Rattus rattus*, PCR, Yucatan

Introduction

The gram-negative spirochete bacteria *Leptospira* spp. is recognized as the causal agent of leptospirosis, an anthropozoonotic disease found on every continent except Antarctica [7]. This disease is most important and frequent in humid tropical or subtropical regions, especially during rainy periods [17]. Previous studies conclude that the risk factors contribute substantially to the increased circulation of *Leptospira* spp. in rural zones where unsanitary conditions generally predominate and present a convergence of the factors, particularly the presence of synanthropic rodents, living near humans [37].

Leptospirosis is currently classed as an emerging disease, due to the occurrence of epidemics in which more than 500,000 serious cases have been reported in humans, with a mortality of 2% to 30% [5]. The World Health Organization (WHO) classifies leptospirosis as a neglected tropical disease and estimates an incidence of 5.1 cases/100,000 people in endemic areas, and 14 cases/100,000 people in epidemics [41]. In Mexico, leptospirosis was first reported in 1920 [39] and it has been considered a public health problem ever since [29]. Compared to other Latin American countries, however, few official reports exist of cases in humans [7]. In the state of Yucatan, different studies have demonstrated the circulation of *Leptospira* spp. among the inhabitants [22, 38, 39].

Leptospira spp., is capable of affecting more than 250 species of domestic and wild mammals [7]. The domestic animals majority that have an infection with *Leptospira* spp. are farm animals, particularly bovines [9, 16, 23] and wild animals are particularly rodents [6, 18, 33]. *Rattus rattus* and *Mus musculus* as the synanthropic rodents, are considered to be the principal reservoirs of *Leptospira* spp. [6, 17, 24], due to the survivability and active multiplication of the germ within their kidney tissue [21]. These species also represent the most important and common route of infectious transmission of the disease to humans. This transmission can be direct or indirect through contact with the urine of infected individuals or with contaminated water, food or soil [3, 4].

Infection in *R. rattus* and *M. musculus* has been reported on numerous occasions [1, 8, 18, 24, 27]. A recently survey of rodents in Kenya, has been conducted to determine the presence of *Leptospira* spp., the PCR analysis showed that 18.3% of rodents carried pathogenic *Leptospira* species in their kidneys, and sequence data identified *L. interrogans* and *L. kirschneri* [11]. However, in Mexico, few studies examine their protagonist role in the dissemination of *Leptospira* spp., illustrating a lack of epidemiological research, especially in the wild or synanthropic mammals of the region [27]. The objective of this study was to use conventional PCR to investigate the condition of reservoir of *Leptospira* spp. in the synanthropic rodents (*M. musculus* and *R. rattus*) captured in a rural community from Yucatan, Mexico, and to characterize the species involved in the infection through sequencing and phylogenetic analysis.

Materials and Methods

STUDY SITE DESCRIPTION

The material of this study was collected from the rural community of Molas, in the state of Yucatan, Mexico (20°40'N, 89°38'W). The predominant vegetation type is tropical low deciduous forest and the climate is warm sub-humid with summer rains (Aw0). The site presents mean maximum and minimum temperatures of 36° of 16°C, respectively. Mean annual rainfall is 1,100mm, and falls mainly from May to October [13]. The village is located within the "Cuxtal" Ecological Reserve.

The community has a population of 2,014 inhabitants and a land area of 30,066 m^2 with 423 households [13]. Most of these dwellings are small, with floors, walls and roofs that are habitually in poor states of repair (with cracks and holes), often lacking in windows and/or doors, and are frequently without toilets. The walls and roofs are generally constructed with cement and blocks of rock, although cardboard, metal, wood, straw and/or clay are also used. The peridomiciliary areas are usually large with abandoned electrical and mechanical equipment and domestic and organic wastes, as well as trees, shrubs and endemic herbs. Animal (birds, pigs and cattle) production is a common activity, and is normally conducted in inadequate installations. Sheep, horses, and domestic or feral dogs and cats are also commonly present.

Selection of households and capture protocol

The site was divided into four quadrants, imagining two perpendicular axes that cross at its center, thus covering the entire urban settlement. In each quadrant, 10 households were chosen for convenience (40 in total) and sampled for three consecutive nights each month. The sampling period was from October 2011 (wet season) to March 2012 (dry season). Trapping effort was 4,320 trap nights per season.

Twelve Sherman traps (7.5cm x 23cm x 9cm, HB Sherman Traps Inc®, Tallahasse, Florida, USA) were set in each household, either distributed at random throughout the interior of the house and in the peridomiciliary area, or in the latter area only depending on the wishes of the owners. Traps were baited with oatmeal and vanilla flavoring, set in the morning and examined the following morning. Traps containing a captured rodent were reset for other and put in the same place.

Sample collection

Capture, management and euthanasia of the rodents was conducted in compliance with the specifications of the American Society of Mammalogists (ASM).

Captured rodents were transferred to the zoology laboratory of the Campus de Ciencias Biológicas y Agropecuarias (CCBA) of the Universidad Autónoma de Yucatán (UADY), where conventional somatic measurements were taken and age and sex determined. After being anaesthetized with ether, the rodents were euthanized by cervical dislocation. Autopsies were conducted in order to collect the organs; both kidneys were removed from each animal and stored at -70°C.

DNA extraction

DNA was obtained from one kidney using the phenol–chloroform method, according to the protocol provided by the suppliers (Trizol™ Reagent, Ambion, Life Technologies, California, USA). The entire organ was macerated with a mortar and pestle that had been pre-sterilized in an autoclave to avoid contamination. Final quantification was obtained with a spectrophotometer (NanoDrop 2000™, Thermo Scientific, Wilmington, USA). Collected DNA (30 µl-40µl) was stored at -70°C until processing.

Polymerase Chain Reaction and phylogenetic analysis

Two PCR reactions were performed. The first utilized the forward primer 16S3 [10] and the reverse primer 16SR [34] in order to amplify a 150 bp segment of the ribosomal 16S gene of *Leptospira* spp. The final reaction volume of 27µl consisted of 3µl of template DNA, 0.5µl of dNTPs, 0.2µl of Taq ADN polymerase (Fermentas™, Waltham, USA), 0.5µl of each primer, 1.5µl of MgCl2, 2.5µl of buffer and 18.3µl of sterile distilled water. The thermocycler parameters were: initial denaturation at 95°C for five min, followed by 34 cycles of a denaturation step at 94°C for 45 sec, an annealing step at 49°C for one min, an extension step at 72°C for two min, and a final extension step at 72°C for five min.

The second reaction was performed only on those extractions that presented a positive result with the first reaction. In this reaction, the reverse primer used was 16S5 [10], while the forward primer was that used in the first reaction. These primers were designed to amplify a 1004 bp segment of the ribosomal 16S gene. The final reaction volume in this case was 28.5µl and the reaction mix deviated

from the first only in that 5µl of template DNA solution was utilized, compared to the 3µl used initially. The cycling parameters were the same as in the first reaction, apart from the temperature in the annealing phase, which was increased to 51°C. The amplicons of this reaction were sent to the company Macrogen™ (World Meridian Venture Center, #60-24, Gasan-dong, Geumchun-gu, Seoul, Korea) for purification, sequencing and phylogenetic analysis. Evolutionary history was inferred using the *Neighbor-Joining* method [30] with the program MEGA5 [36]. Evolutionary distances were calculated using the maximum verosimilarity method [35].

Positive and negative controls were used in both reactions. The former consisted of DNA extracted from the *L. interrogans* serovar *icterohaemorrhagiae* (reference strain), while the latter was sterile water. Products were visualized by electrophoresis on a 1.5% agarose gel and recorded in a gel documentation system.

Statistical analysis

The variables of the captured animals and the PCR results were analyzed with descriptive statistics. In addition, a χ^2 test was used to establish the association (IC 95%, $P<0.05$) between the independent variables (species, sex and age of the rodents) and the results of the PCR (dependent variable). Data were analyzed with the program PASW™ STATISTICS 18 (SPSS Inc. 233, Chicago, IL).

Results

A total of 187 rodents were captured: 57 (30.48%) *R. rattus* and 130 (69.51%) *M. musculus*. All appeared healthy under external physical examination.

As show in figure 1, nine (9/187, 4.81%) individuals were positive for *Leptospira* spp. in the PCR: seven (7/57, 12.80%) *R. rattus* and two (2/130, 1.53%) *M. musculus*. The purification, sequencing and phylogenetic analysis of the ribosomal 16S gene amplicons revealed that the species involved in infection were *L. interrogans* and *L. kirschneri* (figure 2).

We did not find a statistically significant association between the independent variables and the dependent variable ($P>0.05$).

Discussion

This study is the first in the state of Yucatan, Mexico, to provide molecular evidence of infection by *Leptospira* spp. in the species *R. rattus* and *M. musculus* (figure 1) and is the only report (serological or molecular) of infection in the latter specie to date. Infection by *Leptospira* spp. in synanthropic rodents captured in the region has been reported previously by Vado-Solís *et al.* [39], who conducted a seroepidemiological study and found an infection frequency of 15% (9/60) in *R. rattus* by microagglutination test (MAT). The serovars identified by these authors were *wolffi*, *icterohaemorrhagiae* and *bratislava*, belonging to the serogroups *Sejroe*, *Icterohaemorrhagiae* and *Australis*, respectively. These serogroups are found in the pathogenic species *L. interrogans* and *L. kirschneri* [14], which were both identified in the phylogenetic analysis conducted in our study (figure 2). Around the world there are similar studies evaluating pathogenic *Leptospira* species in reservoirs [11].

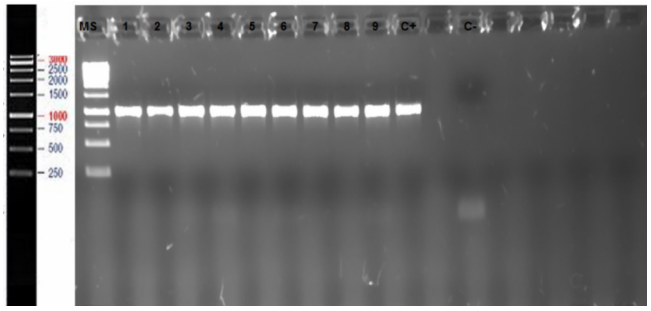

FIGURE 1: Conventional PCR in a 1.5% agarose gel, conducted with DNA extractions, showing the positive products (1004pb) of *Leptospira* spp. MS, molecular size marker. Lines 1-9, positive extractions. Line C+, positive control. Line C-, negative control.

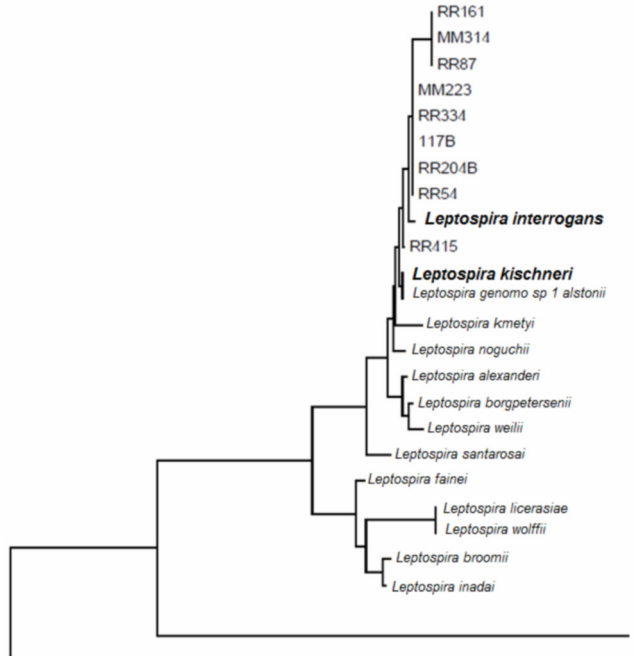

FIGURE 2: Phylogenetic tree based on the sequence of the fragment of the ribosomal 16S gene of *Leptospira* spp. The sequences obtained in our study are shown with the prefixes RR and MM followed by a number. The longitudinal sum of the tree branches is 0.78501417. These are drawn to scale, with branch lengths presented in the same units as those of the evolutionary distances. Sequences were aligned using MEGA5®, and phylogenetic distances were determined by the *Neighbor-Joining* method.

According to the results of the PCR test conducted in our study, both species of rodents are reservoirs of *Leptospira* spp., suggesting that these species could represent an important potential element in the dissemination of the bacteria among the domestic animals and inhabitants of

the community studied [1, 12]. The total prevalence found was 4.81% (9/187), which was lower than that found in the studies of Mgode *et al*. [20] in Tanzania, Agudelo-Flórez *et al*. [1] in Colombia, Rahelinirina *et al*. [27] in Madagascar, Houemenou *et al*. [12] in Benin and Halliday *et al*. [11] in Kenya; but higher than that reported by Gamage *et al*. [9] in Sri Lanka and Langoni *et al*. [16] in Brazil. Percentage of infection was much higher in *R. rattus* (12.28%, 7/57) than in *M. musculus* (1.33%, 2/150), a finding that differs from that found by Perez *et al*. [24] in New Caledonia and Halliday *et al*. [11] in Kenya, but is comparable to that found by Foronda *et al*. [8] in the Canary Islands, Spain.

The total number of individuals that were found to be positive for *Leptospira* spp. in our study was low relative to the number of samples as a direct consequence of the presence of inhibitive elements (degrading enzymes) for the PCR test in the samples of DNA extracted from the kidney tissue [2], since no measures were taken to annul or diminish the effect of these elements. For this reason, it is possible that some infected individuals were not detected. Unfortunately, it was not possible to evaluate the number of positive samples lost. In addition, the prior freezing of the kidney tissue significantly reduces the viability of *Leptospira* spp. [30], implying a negative impact on the quality of DNA extracted and thus affecting the sensitivity and specificity of the PCR [19].

The majority of studies similar to ours reports prevalence values obtained by the measurement of antibodies (mainly by MAT), culture or isolation [3, 15, 26, 33], which hinders direct comparison between those studies and our own; however, many of the prevalence values that have been reported are greater than the percentage of infection found in our study.

The species of *Leptospira* identified in our phylogenetic analysis agree with those reported by Turk *et al*. [38] in Croatia, Houemenou *et al*. [12] in Benin and Halliday *et al*. [11] in Kenya. Gamage *et al*. [9] in Sri Lanka and Rahelinirina *et al*. [27] in Madagascar, only reports the presence of *L. interrogans*. All of these studies were conducted on different synanthropic and/or wild rodents. It is known that *L. interrogans*, *L. kirschneri* and *L. borgpetersenii* cause the majority of the cases of human leptospirosis worldwide [4, 6, 11].

There was no significant statistical association (P>0.05) between the independent variables of the rodents and their condition as reservoirs of *Leptospira* spp. (positive result in the PCR) to indicate a particular relationship between these factors and the probability of transmission of *Leptospira* spp. However, it has been widely reported that the synanthropic rodents are the main reservoirs of *Leptospira* pathogens, and that their mere presence represents a considerable risk factor in terms of the transmission of leptospirosis [9].

Leptospirosis is found worldwide and presents a higher number of clinical cases in tropical regions [37], its ecology involves a complex interaction between humans, reservoir and host animals, the etiological agent and the environment in which these coexist [4]. This zoonotic disease is normally described as being most frequent in locations with inadequate sanitation and poor hygiene practices, where ideal conditions exist for proliferation of reservoirs (especially synanthropic rodents) with which humans have close links or contact [32]. Perret *et al*. [25], also indicate that the most important risk factor in terms of acquiring infection is rodent infestation associated with poor living conditions, characteristics that predominate in the majority of households present in the study area.

Our study demonstrated the presence of pathogenic *Leptospira* species in the kidney tissue of synanthropic rodents captured in Yucatan, Mexico, a finding that qualifies these animals as reservoirs of the bacteria germ. The rodents are infected with *L. interrogans* and *L. kirschneri* and thus present a potential risk of transmission of the agent to domestic animals and humans in the study area.

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