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Sección Especial

LOS MAMÍFEROS COMO HOSPEDADORES DE PARÁSITOS

Artículo

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MARSH DEER (*Blastocerus dichotomus*) AS A NEW HOST FOR *Leptospira borgpetersenii* IN ARGENTINA

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ABSTRACT. Leptospirosis is a worldwide re-emerging zoonosis caused by bacteria of the genus *Leptospira*. All mammals are potentially susceptible to pathogenic *Leptospira*, while certain species can act as reservoirs of the bacterium, whose main route of transmission is water. The marsh deer (*Blastocerus dichotomus*) is a wetland-dependent native cervid distributed in the Paraná-Paraguay River and influence areas. During the last decades, its populations have undergone mortality episodes of multifactorial origin. Since wetlands constitute favorable scenarios for the transmission of *Leptospira*, we investigated the occurrence of this agent in 12 marsh deer from the subpopulation of the Paraná Delta in Argentina, categorized as "Endangered". DNA was extracted from urine and/or kidney samples, and characterization was performed at the species level by PCR amplification of the 16S rRNA gene. An aliquot of urine and serial dilutions of kidney macerates were seeded in a semisolid EMJH medium spiked with 5-Flurouracil, and isolation was achieved in a kidney sample. *Leptospira borgpetersenii* was detected in two samples (one from urine and one from kidney), constituting the first report in marsh deer. Our findings add a new species as the host of *L. borgpetersenii*, whose circulation in wildlife has been little studied in the region. Further studies are needed to determine the transmission patterns of this bacterium in wildlife, evaluate its pathogenicity in marsh deer and other wild species, and explore the existence of reservoirs in natural areas.

RESUMEN. EL CIERVO DE LOS PANTANOS *Blastocerus dichotomus*) COMO UN NUEVO HOSPEDADOR DE *Leptospira borgpetersenii* EN ARGENTINA. La leptospirosis es una zoonosis reemergente extendida a nivel mundial causada por bacterias del género *Leptospira*. Todos los mamíferos son potencialmente susceptibles a las variantes patogénicas de *Leptospira*, mientras que determinadas especies pueden actuar como reservorios de la bacteria, cuya principal vía de transmisión es el agua. El ciervo de los pantanos (*Blastocerus dichotomus*) es un cérvido nativo dependiente de humedales que se distribuye

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en el corredor fluvial Paraná-Paraguay y áreas de influencia. Durante las últimas décadas, sus poblaciones atravesaron episodios de mortalidad de origen multifactorial. Dado que los humedales constituyen escenarios propicios para la transmisión de *Leptospira*, hemos investigado la ocurrencia de este agente en 12 ciervos de los pantanos de la subpoblación del Delta del Paraná en Argentina, categorizada como "En Peligro". Se extrajo ADN de muestras de orina o riñón, y se realizó la caracterización a nivel de especie mediante amplificación por PCR del gen *16S rARN*. Una alícuota de orina y diluciones seriadas de macerados de los riñones se sembraron en un medio semisólido EMJH adicionado con 5-Flurouracilo, y se logró el aislamiento en una muestra de riñón. Se detectó *Leptospira borgpetersenii* en dos muestras (una de orina y otra de riñón), lo que constituye el primer informe de esta especie en ciervo de los pantanos. Nuestros hallazgos añaden una nueva especie como hospedador de *L. borgpetersenii*, cuya circulación en la fauna silvestre ha sido poco estudiada en la región. Se necesitan nuevos estudios para determinar los patrones de transmisión de esta bacteria en la fauna silvestre, evaluar su patogenicidad en el ciervo de los pantanos y en otras especies silvestres, y explorar la existencia de reservorios en áreas naturales.

Palabras clave: Fauna silvestre, humedales, leptospirosis, reservorios de enfermedades infecciosas, zoonosis.
Key words: Leptospirosis, reservoirs of infectious diseases, wildlife, wetlands, zoonoses.

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INTRODUCTION

The marsh deer (*Blastocerus dichotomus*), the biggest cervid of South America, inhabits wetlands and marshy habitats in east-central and north-eastern Argentina, west-central and southern Brazil, Paraguay, south-eastern Peru, and eastern Bolivia (Pinder & Grosse 1991; Piovezan et al. 2010). Listed as "Vulnerable" by the International Union for Conservation of Nature (IUCN) (Duarte et al. 2016) and the Red List of Mammals of Argentina (Pereira et al. 2019), the main threats to its conservation are habitat loss, hunting, dog attacks, and diseases (Pinder & Grosse 1991; Piovezan et al. 2010; Duarte et al. 2016; Pereira et al. 2019). In Argentina, the distribution range of this species is fragmented into four subpopulations: Esteros del Iberá, Delta del Paraná, Formosa, and Humedales del Paraná Medio, which have been assigned different conservation categories (Pereira et al. 2019). Mortality events of marsh deer have been recorded in the Esteros del Iberá subpopulation in Argentina since the 1990s (Beccaceci 1994; Orozco et al. 2013, 2020; Orozco & Di Nucci 2023). However, it is only in recent decades that these phenomena have been studied in depth, revealing their multifactorial origin and suggesting an association between potentially pathogenic agents and environmental, ecological, and epidemiological factors (Orozco et al. 2013, 2020; Orozco & Di Nucci 2023).

During the extraordinary floods in 2016, a large number of marsh deer died in the Delta del Paraná subpopulation (Pereira et al. 2023). While the major cause of death was hunting (Pereira et al. 2023), marsh deer with poor body condition, high tick burdens, infection with vector-borne pathogens, and the presence of harmful gastrointestinal parasites were found in the study area (Orozco et al. 2020). Coinfections in wildlife were found with high frequency and would increase the mortality risk under stressful situations, and adverse meteorological conditions may trigger parasitism with clinical symptomatology (Duarte 1997; Pedersen & Greives 2008; Watson 2013).

Leptospirosis is a worldwide zoonotic disease caused by spirochetes from the genus *Leptospira* (Levett 2001). According to their pathogenicity, they can exist as non-infectious environmental saprophytes or can cause infections that vary in severity from asymptomatic carriage to acute infection in both humans and animals (Picardeau 2017). The genus comprises 65 species that were recently reclassified into two groups, pathogenic (P) and saprophytic (S), and further subdivided into P1 and P2, and S1 and S2, respectively (Vincent et al. 2019). Earlier classifications divided the genus into more than 300 serovars (sv), which are defined according to structural differences in the carbohydrate component of their lipopolysaccharide (LPS). Pathogenic *Leptospira* serovars generally have specific host preferences: sv. Hardjo and cattle, sv. Canicola and dogs, and sv. Icterohaemorrhagiae and rats, but these associations are not absolute, and the associations

of different serovars with wild animals remain unknown (Picardeau 2017). *Leptospira* spp. is transmitted by direct contact with infected animals or by indirect contact in environments contaminated with infected urine (Levett 2001). *Leptospira* spp. can infect a wide variety of species, including domestic and wild animals. Rodents are considered its main reservoirs, and they were also found in opossums, armadillos, foxes, coatis, capybaras, bats, deer, and pinnipeds (Levett 2001; Cameron et al. 2008; Dubay et al. 2015; Albuquerque et al. 2017; Fornazari et al. 2018; McCutchan et al. 2023). In Argentina, antibodies against *Leptospira* spp. were reported in pampas deer (*L. interrogans* serovars hardjo, mini, wolffi, and pomona) and marsh deer (*L. interrogans* serovar pyrogenes) (Uhart et al. 2003; Orozco et al. 2020).

The aim of this study was to investigate the presence of pathogenic *Leptospira* in marsh deer sampled in the Delta del Paraná during a mortality event associated with scenarios conducive to *Leptospira* transmission, such as extreme flooding.

MATERIALS AND METHODS

Fieldwork was conducted in the Delta del Paraná subpopulation during the extraordinary floods that occurred between late 2015 and 2016. The area (14,000 km^2) is part of the Paraná Delta Biosphere Reserve, located in the Paraná River floodplain in the provinces of Buenos Aires and Entre Ríos (34°15'S, 58°58'W). It comprises the typical deltaic morphology with a permanent additional growth of alluvial lands on the outer front of the Paraná River. For more than 150 years, it was subjected to intensive forestry associated with the construction of dams and roads. At present, subsistence and sport hunting are frequent, although illegal. The climate is temperate, with average temperatures of around 16-18 °C and an annual rainfall of 1,073 mm (Kandus & Malvárez 2004).

The capture and handling procedures for live marsh deer were described in detail elsewhere (Orozco et al. 2020). Biosafety and animal processing procedures were performed according to approved protocols (Argentinean Institutional Committee for the Care and Use of Experimental Animals; Protocol N° 2014-40), issued by the Faculty of Veterinary Sciences, University of Buenos Aires. Capture and transit permits were obtained from the provincial government.

Blood samples were collected by jugular vein puncture (10-15 mL, live individuals) or cardiac puncture (15-20 mL, dead individuals). An aliquot of blood was centrifuged, and serum was stored at -80 °C. Serological diagnoses using microscopic agglutination tests (MAT, serovars *ballum, castellonis, canicola, grippotyphosa, icterohaemorrhagiae, copenhageni, pomona, pyrogenes, sejroe, wolffi, and tarassovi*; *cut-off* value 1:50) were performed by the National Service of Agri-Food Health and Quality (SENASA) according to the procedures described by the World Organization for Animal Health (OIE 2015), and the results were informed elsewhere (Orozco et al. 2020). Two live marsh deer showed evidence of exposure to *Leptospira interrogans* serovar *pyrogenes* (titers 1/200 and 1/100) (Orozco et al. 2020).

During necropsies, all organs were evaluated macroscopically. Kidney and liver samples were collected, including part of normal tissue and part of injured tissue, if present. One fragment of each tissue was fixed in a 10% buffered formalin solution (BFS), and another was frozen at -80 °C. Where possible, a urine sample was collected during voluntary urination in live individuals or vesical puncture in dead individuals and stored at 4 °C.

Samples fixed in BFS were processed using conventional histopathological protocols (Bancroft & Gambl 2002). Tissues were embedded in paraffin wax, and 5 μm sections were obtained, which were stained with hematoxylin and eosin.

DNA was extracted from frozen kidney samples using Tri-Reagent (Ambion, USA). Briefly, a portion of the organ was homogenized in 1 mL of Tri-Reagent and the homogenate was used for DNA extraction following the instructions of the manufacturer. DNA extraction from urine samples was performed using the DNeasy Blood & Tissue Kit (Qiagen, USA), following the instructions of the manufacturer. PCR reactions were performed in a final volume of 50 μL. Species identification was determined using the set of primers described by Mérien and colleagues for 16S rRNA amplification: (LA: 5´-GGCGGCGCGTCTTAAACATG-3´ and LB: 5´-TTCCCCCCATTGAGCAAGATT-3´) (Mérien et al. 1992). The PCR cycling conditions consisted of an initial denaturation step of 94 °C for three min, followed by one cycle of annealing at 54 °C for 90 sec an extension step of 72 °C for two min (one cycle); 29 cycles of denaturation step at 94 °C for one min, annealing at 54 °C for 90 sec, and extension at 72 °C for two min, and a final extension step at 72 °C for 10 min. DNA from the reference strain *Leptospira interrogans* serovar Copenhageni Fiocruz L1-130 was used as a positive control and H_2O dd as a negative control. Sequencing of PCR products was performed in the Genotyping and Sequencing facility of the Biotechnology Institute (INTA), and the 16S rRNA consensus sequences were analyzed using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

For culture, approximately 50 mg of the kidney was homogenized using a mechanical tissue homogenizer. Of these homogenates, 1 mL was used to inoculate 10 mL of EMJH liquid media. Cultures were prepared in duplicate, incubated at 30 °C, and checked weekly for development. Positive cultures were subcultured in 10 mL of EMJH. Three mL of these cultures were used for DNA extraction using Tri-Reagent, as mentioned before. One μL of DNA was used for 16S-rRNA amplification.

RESULTS

A total of 12 kidney samples and three urine samples were obtained from 12 marsh deer; only one urine sample was collected from a live animal. One urine sample and one kidney sample from a different deer were PCR-positive. The recovery rate of the isolates was 8% (1/12) from the same positive kidney sample. Sequencing of the *16S rRNA* gene determined that in both cases the species identified was *Leptospira borgpetersenii* (**Fig**. 1). A phylogenetic relationship tree

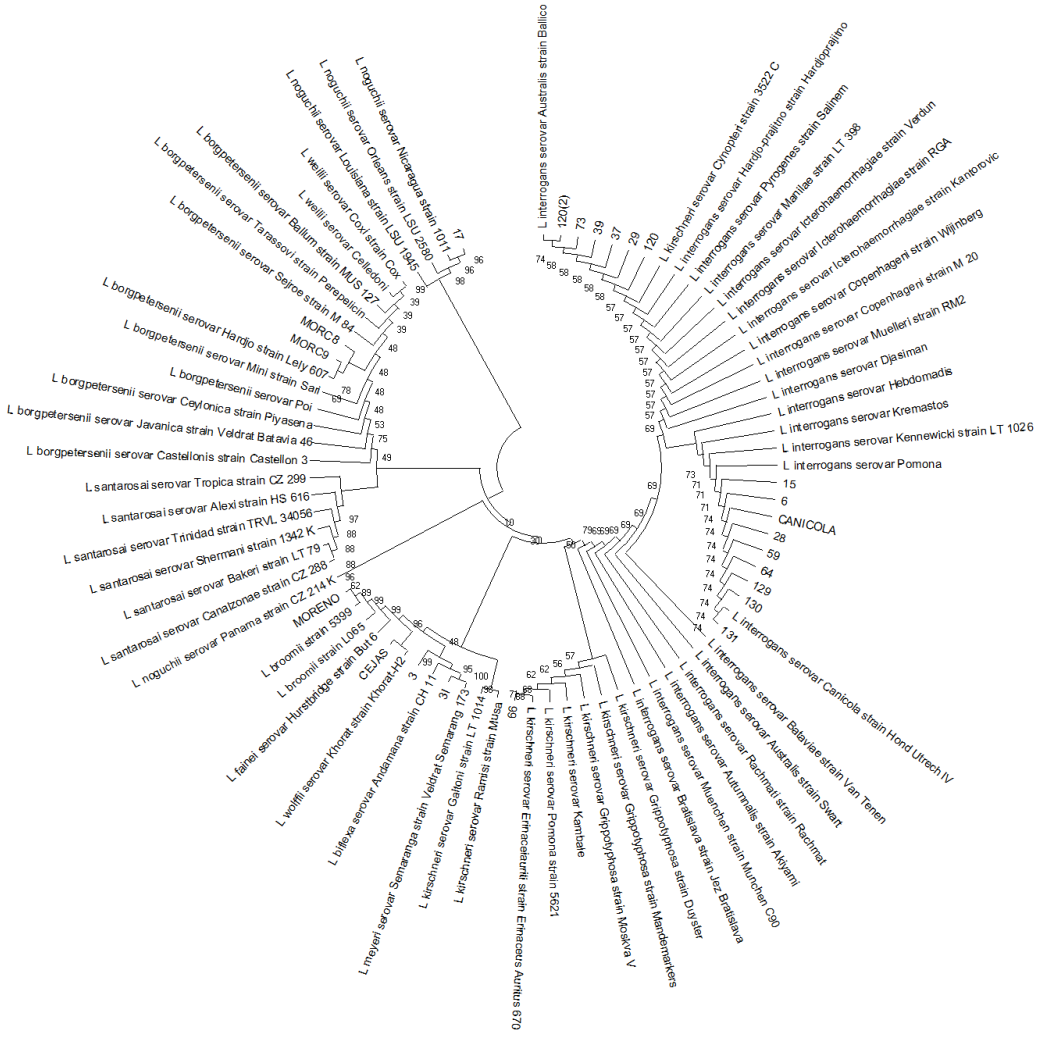

Fig. 1. Maximum likelihood tree based on 16S rRNA sequences. Phylogenetic relationships were inferred using the maximum likelihood method and the Tamura-Nei model (500 bootstrap replicates, MEGA X). Samples from Marsh Deer are MORC8 and MORC9, respectively.

using 16S rRNA sequences from different *Leptospira* species confirmed this result (**Fig**. 1). Sera from both animals was negative by MAT (Orozco et al. 2020).

No particular lesion was observed in the kidneys of marsh deer by histopathological analysis. In the liver tissue, mild inflammatory infiltrates were detected in the portal spaces, consisting of lymphocytes and occasional polymorphonuclear infiltrates. A small focal inflammatory infiltrate could be identified in the liver parenchyma, adjacent to a portal space, characterized by lymphocytes, both small and medium-sized, and macrophages, separating the hepatocytes.

DISCUSSION

Our study documents the first isolation and molecular characterization of *Leptospira borgpetersenii* in marsh deer, a threatened native mammal from Argentina.

Leptospira borgpetersenii is a pathogenic spirochete considered a re-emerging zoonotic agent. Together with *L. interrogans* are the two main causes

of human leptospirosis worldwide (Barbagelata et al. 2013). In South America, it has also been found in domestic and wild animals (Colombo et al. 2018; Zarantonelli et al. 2018), being isolated in free-living white-eared opossum (*Didelphis albiventris*) in Brazil (Jorge et al. 2012), and in aborted wild boar (*Sus scrofa*) fetuses in Argentine Patagonia (Brihuega et al. 2017). In the Delta del Paraná, *L. borgpetersenii* was recently isolated from the rodent *Scapteromys aquaticus* (Colombo et al. 2018), which overlaps its habitat with marsh deer and domestic livestock, sharing soil and water resources in the study area.

Although the clinical symptoms of infection in humans due to both pathogenic *Leptospira* species are similar, epidemiological data show different transmission patterns between them. Some authors describe a host-to-host transmission cycle for *L. borgpetersenii* associated with limited survival in the environment compared to *L. interrogans*, due to an inability to acquire nutrients in an environment external to its mammalian hosts (Bulach et al. 2006; Picardeau et al. 2008). Our finding of *L. borgpetersenii* in a solitary species such as the marsh deer (Pereira et al. 2019), which naturally has reduced contact between individuals, could be in contrast with the epidemiological theory that supports direct host-host transmission for this species. However, the context of this finding corresponds to an uncommon environmental scenario, characterized by extreme flooding that reduced the suitable habitat, forcing animals of different species to share the scarce and small dry patches (Orozco et al. 2013, 2020; Orozco & Di Nucci 2023), which could have favored the transmission of *L. borgpetersenii* between deer.

Although the circulation of *L. borgpetersenii* could be low in the study area, the transmission patterns of this bacterium and the low temperatures during field-work (unsuitable for the development of pathogenic leptospires) could explain our results. Increased sampling of marsh deer and rodents in the study area, including a seasonal design, is needed to better understand the ecoepidemiology of *L. borgpetersenii* in the Delta del Paraná.

Information on leptospirosis in free-ranging marsh deer is extremely scarce. Only a few serological studies in Brazil (Galli et al. 2014) add to our previous serological survey in Argentina (Orozco et al. 2020; Orozco & Di Nucci 2023). Here, we report the absence of lesions in renal tissues associated with nonspecific inflammatory lesions in the liver of the only positive dead marsh deer. These findings do not allow for concluding the pathogenicity of *Leptospira* infection in marsh deer. Further studies of this pathogen in marsh deer are required to establish possible associations between *Leptospira* infection and tissue lesions. Monitoring of *Leptospira* in marsh deer could alert about the re-emergence of *Leptospira* serovars in the area, contributing to wildlife disease surveillance and biodiversity conservation.

CONCLUSIONS

Our findings add a new mammal species as a host of *L. borgpetersenii*, a zoonotic and re-emerging bacterium poorly studied in the region. Our results provide evidence of the circulation of this bacterium in natural environments in Argentina and raise new questions about its transmission patterns and the risk of infection for humans and other species. Further studies are needed to assess the pathogenicity of *L. borgpetersenii* in marsh deer and to establish its role in mortality events as a conservation threat.

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