Feedback on unanswered questions - Tracking *Leptospira* sources

Do you recommend extrapolating clinical and epidemiological data from humans to primates in captivity?

Non-human primates appear to be susceptible to Leptospira, particularly L. sg Icterohaemorrhagiae, similar to humans (see the attached paper). The associated clinical picture is comparable to that in humans (jaundice, hemorrhages, etc.). However, although some similarities have been reported, extrapolation of the observations in humans remains limited. In our laboratory experience, seroconversion in non-human primates is lower than in humans. We typically use lower positive thresholds from 1:10 and recommend monitoring antibody kinetics where possible.

See article below.

Beato-Benítez A, Cano-Terriza D, Gonzálvez M, Martínez R, Pérez-Cobo I, Ruano MJ, Guerra R, Mozos-Mora E, García-Bocanegra I. Fatal leptospirosis in endangered Barbary macaques (Macaca sylvanus) kept in captivity: Assessing the role of sympatric rodents. Vet Microbiol. 2024 Apr;291:110028. doi: 10.1016/j.vetmic.2024.110028. Epub 2024 Feb 14. PMID: 38367538.

Why is the lesion created in kidneys during leptospiruria of the type interstitial nephritis, not nephritis? While we know Leptospira are present inside kidney tubules.

Nephritis is a general term for inflammation of the kidney but depending on the infectious agent and the pathogenic process, and therefore the distribution of lesions and the type of inflammation, there may be glomerulonephritis with a focus on the glomerulus, interstitial nephritis, pyelonephritis, etc., which are "subcategories" of nephritis. Tubulointerstitial nephritis due to Leptospira is explained by the migration of bacteria from the bloodstream into the renal tubules, with endothelial damage, interstitial inflammation, and tubular damage along the way (it may be neutrophilic and/or lymphoplasmacytic). It is important for the pathologist to be precise in the characterization of the lesions. The observation of microscopic lesions of tubulointerstitial nephritis leads us to leptospirosis.

(c) Rozenn Le Net, Pathologist

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Fatal leptospirosis in endangered Barbary macaques (*Macaca sylvanus*) kept in captivity: Assessing the role of sympatric rodents

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ABSTRACT

Between December 2020 and January 2021, an outbreak of acute mortality in endangered Barbary macaques (*Macaca sylvanus*) kept in captivity was detected in a zoo in Spain. The main findings observed in the two fatally affected animals at *post-mortem* evaluation were jaundice, renal tubular necrosis and interstitial nephritis. *Leptospira* spp. infection was confirmed by real time PCR (qPCR) in different tissues in both individuals. Analyses of *sec*Y gene from a positive individual showed 100% homology with a previously published sequence corresponding to *Leptospira interrogans* serovar Copenhageni. Free-living sympatric brown rats (*Rattus norvegicus*) from the affected zoo were also analyzed, and showed a prevalence and seroprevalence of *Leptospira* spp. of 18.2% (4/22; 95% CI: 2.1–34.3) and 41.9% (26/62; 95% CI: 29.7–54.2), respectively. We detected seropositive sera to five different serovars of *Leptospira* spp. (Copenhageni, Grippotyphosa, Pomona, Canicola and Hardjo) in the rodent population, with *L.* Copenhageni being the predominant one. This study describes for first time an outbreak of fatal leptospirosis in captive non-human primates in Europe. Our results show that Barbary macaques, an endangered species, are highly susceptible to *Leptospira* spp. infection, with sympatric wild rodents being the most likely reservoir animals involved in transmission in this outbreak. Our results suggest that rodent control could be an effective measure for minimizing exposure to *Leptospira* spp. in zoological collections. Given the potential implications for conservation, animal and public health, non-human primates and rodents should be included in surveillance programs for *Leptospira* spp. in zoos.

1. Introduction

Leptospirosis is a worldwide zoonotic disease caused by pathogenic bacteria of the genus *Leptospira* (order *Spirochaetales*, family *Leptospiraceae*) (Adler and de la Peña Moctezuma, 2010). This notifiable disease to the World Organization for Animal Health (WOAH) is of public and animal health concern, causing about 60,000 human deaths per year and severe economic losses in livestock production (Costa et al., 2015; Ellis, 2015; WOAH, 2021). Transmission of *Leptospira* spp. occurs mainly through direct or indirect contact with the blood or urine of infected individuals (Bharti et al., 2003; Murray, 2015).

Leptospira spp. currently comprise 66 pathogenic and non-pathogenic species (Vincent et al., 2019; Casanovas-Massana et al., 2020), including more than 300 different leptospiral serovars (Adler and de la Peña Moctezuma, 2010). This taxonomic variety allows *Leptospira* spp. to infect more than 160 mammal species, with rodents as key natural reservoirs hosting a wide variety of *Leptospira* serovars (Langston and Heuter, 2003; Millán et al., 2009; Ullmann and Langoni, 2011;

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Krijger et al., 2019). As these micromammal species are ubiquitous worldwide, they have been involved in outbreaks of leptospirosis in a wide variety of susceptible species, including humans (Adler and de la Peña Moctezuma, 2010; Boey et al., 2019). Rodents have been associated with *Leptospira* spp. infections in domestic, free-living, and captive animal species (Zenebe et al., 2013; Cilia et al., 2021; Hardgrove et al., 2021). In captive wildlife, *Leptospira* spp. have previously been associated with mortality outbreaks in non-human primates (NHPs) in zoos in the Americas (Shive et al., 1969; Reid et al., 1993; Baitchman et al., 2006; Szonyi et al., 2011; González et al., 2015; Wilson et al., 2021). In Europe, however, there is limited information on the role of captive NHPs and sympatric rodents in the epidemiology of *Leptospira* spp. (Ferreira et al., 2020; Hardgrove et al., 2021). In the present study, we report a fatal outbreak of leptospirosis in captive Barbary macaques (*Macaca sylvanus*), a species listed as endangered on the IUCN Red List (Wallis et al., 2020), at a zoo park in Spain. We also assessed the epidemiological role of free-living rodents at the affected zoo as a possible source of transmission of *Leptospira* spp. in this outbreak.

2. Material and methods

Between December 2020 and January 2021, an outbreak of acute mortality in two adult female Barbary macaques was detected at the Cordoba Zoo Conservation Center (CZCC) in Spain. The affected individuals presented anorexia and apathy, and died within 24 hours after the appearance of the first clinical signs. Both animals were sent to the Faculty of Veterinary Sciences of the University of Cordoba (southern Spain) for a systematic and detailed *post-mortem* examination. At necropsy, duplicate tissue samples (lungs, heart, liver, spleen, kidneys and urinary bladder) were collected from both specimens for laboratory analysis; one aliquot of the sample was preserved under freezing conditions (−20°C) for future molecular analyses, and the other was fixed in 10% neutral phosphate-buffered formalin for 24 h at room temperature. The fixed samples were embedded in paraffin wax and stained with standard hematoxylin and eosin stain (H and E). A blood sample was taken from the intracranial venous sinuses of one macaque (Jiménez-Ruiz et al., 2016). The blood was centrifuged at 3000 *g* for 10 min to obtain serum, which was stored at −20°C until analysis.

A total of 71 free-living sympatric rodents were also sampled at the affected zoo between 2017 and 2021. The rodents were captured as part of the official rodent control program carried out by the local authorities, following the European standard specifying the requirements and competences to be met by professional pest management services (UNE 16636). The animals were captured using Tomahawk live traps, which were checked every 12 hours, then anesthetized by intramuscular injection of medetomidine (1 mg/kg) combined with ketamine (50 mg/kg) which allowed manipulation to obtain blood samples from the coccygeal veins (Mayer and Mans, 2018). The rodents were euthanized by intracardiac puncture with sodium pentobarbital (>150 mg/kg) (Mayer and Mans, 2018). All the specimens were identified as brown rats (*Rattus norvegicus*), following guidelines by MacDonald, Barrett (2008). Blood samples from 62 of the 71 rats were collected and centrifuged at 3000 *g* for 10 min to obtain serum. In addition, during *post-mortem* examination, a set of tissues (liver, kidneys and urinary bladder) were also collected from 22 of the 71 rodents. All rodent sera and tissue samples were stored at −20°C until analysis.

2.1. Laboratory analysis

Serum samples from the Barbary macaques and brown rats were sent to the Spanish National *Leptospira* Reference Laboratory in Algete (Madrid, Spain) to determine the seroprevalence of *Leptospira* spp. by the microscopic agglutination test (MAT), considered the reference test method by the WOAH (2021). Briefly, the test was carried out in two successive steps. The first involved a screening test, in which each serum in 1:30 dilution was screened against live strains of leptospires for *Leptospira* serovars Hardjo, Canicola, Grippotyphosa, Copenhageni, Bratislava and Pomona, representative of the serogroups known to circulating in Spain (Alonso-Andicoberry et al., 2001a, 2001b). In the second phase, sera positive in the screening dilution of 1:30 underwent a titration process against the specific strain serovars in consecutive dilutions of 1:10, 1:30, 1:100, 1:300, 1:1000 and 1:30,000.

DNA from blood and tissues was extracted using automated BioSprint 96 DNA Blood Kit system (QIAGEN®, Hilden, Germany) and ATL buffer, according to the manufacturer's recommendations. Detection of the *lipL*32 gene, present only in pathogenic *Leptospira* species, was performed by qPCR using primers LipL32—45 F (5′-AAGCATTACCGCTTGTGGTG-3′), LipL32—286 R (5′-GAACTCCCATTTCAGCGATT-3′) and LipL32–189 P (FAM-5′-AAAGCCAGGACAAGCGCCG-3′-BHQ1), which generates a 242-base pair fragment, as previously described (Stoddard et al., 2009).

For the molecular characterization of *Leptospira* spp., the *sec*Y gene, encoding preprotein translocase for *Leptospira* spp., was amplified by PCR in positive samples using the primers SecYF (5′-ATGCCGATCATTTTTGCTTC-3′) and SecYR (5′-CCGTCCCTTAATTTTAGACTTCTTC-3′) and nested primers SecYIVF (5′-GCGATTCAGTTTAATCCTGC-3′) and SecYIV (5′- CTTAGATTTGAGCTCTAACTC-3′) with a target of 202 base pairs (Almeida et al., 2019). PCR products were purified using the QIAquick PCR & Gel Cleanup Kit (QIAGEN, Spain) and sequenced. The sequence was assembled, edited and aligned using the MUSCLE tool in MEGA software, version 11 (Tamura et al., 2021). It was further compared with published *Leptospira* sequences using online BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited in GenBank, using the National Center for Biotechnology Information (NCBI) BankIt v3.0 submission tool (NCBI, 2023).

3. Results

Two of seven Barbary macaques living in the same facility were affected during the outbreak (29% morbidity and mortality rate, 100% fatality rate). No clinical signs were observed in the remaining individuals. At necropsy, both Barbary macaques were in good body condition. Generalized jaundice was the main lesion observed in several organs and tissues, including the skin, oral mucosa, subcutaneous tissues, heart, lungs, serosa of the abdominal cavity and kidneys (Fig. 1A-E). Acute-subacute congestion was also found in the lungs, liver, spleen and kidneys (Fig. 1E).

Histopathological analysis revealed acute renal tubular necrosis in the proximal and distal convoluted tubules, as well as mild to moderate chronic interstitial nephritis, characterized by mononuclear inflammatory infiltrates (Fig. 1F). Acute multifocal hemorrhages, generalized congestion and edema were also observed in the heart, lungs, liver, spleen and kidneys.

No specific antibodies against *Leptospira* spp. were found in the dead Barbary macaque tested by MAT. Seroprevalence in the sympatric brown rat population was 41.9% (26/62; 95% CI: 29.7–54.2). In seropositive rats, the most prevalent agglutinated serovar was *L.* Copenhageni (73.1%; 19/26), with titers ranging between 1:30 and 1:1000, followed by *L.* Grippotyphosa (38.5%; 10/26), *L.* Pomona (28.0%; 7/25), *L.* Canicola (15.0%; 3/20) and *L.* Hardjo (4.8%; 1/21) (Table 1).

The presence of *Leptospira* spp. DNA was confirmed in the kidneys of both affected Barbary macaques and in the blood, liver and urinary bladder of one of them. With respect to the brown rats, *Leptospira* spp. infection was detected in four of the 22 (18.2%; 95% CI: 2.1–34.3) specimens analyzed by qPCR. *Leptospira* spp. DNA was found in the kidneys (26.7%; 4/15) and urinary bladder (9.1%; 1/11), but not in the liver (0.0%; 0/22). Two of the four qPCR-positive rodents tested by MAT had specific antibodies against serovar *L.* Copenhageni (1:300) (Table 1).

Only qPCR-positive samples with Ct < 34 (blood and kidneys from one of the dead macaques) were further characterized by PCR amplification and sequencing of the *sec*Y gene. The sequence obtained from this

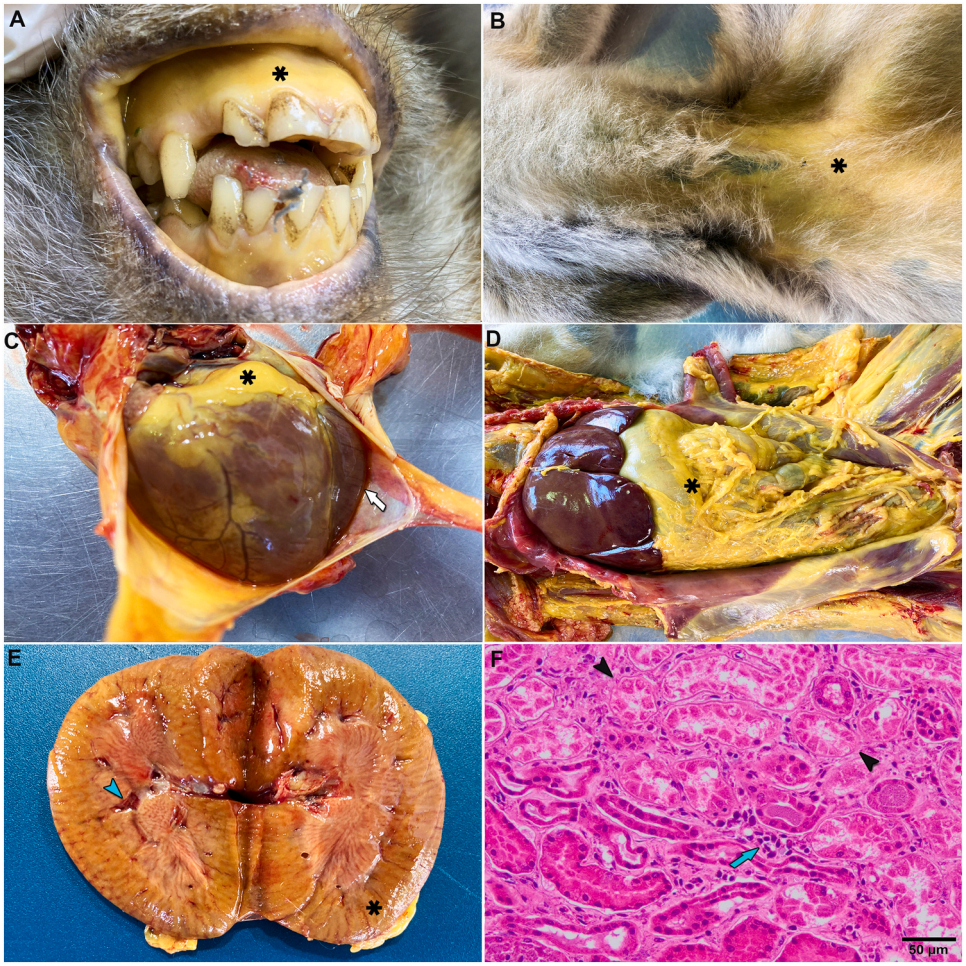

Fig. 1. Main macroscopic and histopathologic findings in the affected Barbary macaques: A) Yellow discoloration of the oral mucosa indicating jaundice (asterisk). B) Icteric skin (asterisk). C) Jaundiced heart (asterisk) and mild hydropericardium (white arrow). D) Serosa of the abdominal cavity with yellowish discoloration because of jaundice (asterisk). E) Kidney marrow with congestion (blue arrowhead) and renal cortex with yellow discoloration due to jaundice (asterisk). F) Kidney section stained with hematoxilin-eosilin (H and E) showing acute renal tubular necrosis in the proximal and distal convoluted tubules (black arrowhead) and interstitial nephritis characterized by mononuclear inflammatory infiltrate (blue arrow).

individual was deposited in GenBank under accession number OR224347. PCR products showed 100% homology with a previously published sequence of *L. interrogans* serovar Copenhageni (GenBank accession number CP048830).

4. Discussion

To the best of the authors' knowledge, this is the first study describing mortality from *Leptospira* spp. infection in captive NHPs in Europe. There is only one previous report on fatal leptospirosis in Barbary macaques at a zoo in the United States, which was associated with *Leptospira* serovar Icterohaemorrhagiae infection (Shive et al., 1969). The clinical signs observed in the affected macaques (anorexia and apathy) were similar to those previously reported in other captive NHP species in the Americas (Baitchman et al., 2006; Szonyi et al., 2011; González et al., 2015; Wilson et al., 2021). With regard to the lesions, the main macroscopic (generalized jaundice) and histopathologic findings (renal tubular necrosis and interstitial nephritis) also coincided with those previously reported in Barbary macaques and other NHP species belonging to the families Cebidae, Atelidae and Callitrichidae in New World countries (Shive et al., 1969; Szonyi et al., 2011; González et al., 2015; Wilson et al., 2021).

The rapid clinical evolution (less than 24 hours) of the affected macaques, as well as the absence of antibodies against *Leptospira* spp. in the individual that was serologically tested, were consistent with an acute process of leptospirosis. Previous studies have shown that anti-*Leptospira* spp. antibodies can be detected between six and 11 days after infection in different NHP species experimentally infected with different serovars of *L. interrogans* (Palmer et al., 1987; Pereira et al., 2005). Our results indicate that the Barbary macaque is highly susceptible to *Leptospira* spp. infection.

With respect to the brown rats, the seroprevalence (41.9%) and prevalence of infection (18.2%) values found indicate a high circulation of *Leptospira* spp. in the sympatric rodent population of the zoo. A similar prevalence of infection (15.1%; 19/126) was found in the same rodent species in a zoo in Germany (Heuser et al., 2017). The serological results also coincided with those reported in brown rats sampled in two Brazilian zoos (25.6%; 11/43) (Pellizzaro et al., 2017), but were lower than those reported in black rats (*Rattus rattus*) from a zoo in Colombia (66.7%; 10/15) (Woolf et al., 2021). By contrast, seropositivity was lower in a limited number of brown rats from a zoo in the Czech Republic (17.0%; 1/6) (Pittermannová et al., 2021). Although further studies involving larger numbers of animals are warranted, all these studies highlight rodents as relevant reservoirs of *Leptospira* spp. in zoos.

The high circulation of *Leptospira* spp. in the sympatric rat population supports the hypothesis that rodents acted as transmission reservoirs in this outbreak, as has been previously suggested (Szonyi et al., 2011). This result is in agreement with previous surveys confirming the key role

Table 1
Microscopic agglutination test (MAT) results on positive sera samples of 26 free-living sympatric brown rats (*Rattus norvegicus*) captured between 2017 and 2021 at the Cordoba Zoo Conservation Center (CZCC) in Spain. Red and green triangles indicate seropositivity and seronegativity to each *Leptospira* serovar tested. Antibody titers are shown in parentheses.

ID	Specie	*L.* Hardjo	*L.* Canicola	*L.* Grippotyphosa	*L.* Copenhageni	*L.* Bratislava	*L.* Pomona
1	Brown rat	▲	–	▲	▲ (1:300)	–	▲
2	Brown rat	–	–	▲ (1:30)	▲ (1:100)	–	▲ (1:30)
3	Brown rat*	▲	▲	▲	▲ (1:300)	▲	▲
4	Brown rat*	▲	▲	▲	▲ (1:300)	▲	▲
5	Brown rat	–	▲ (1:100)	▲ (1:100)	▲	▲	▲
6	Brown rat	▲	▲	▲	▲	▲	▲ (1:100)
7	Brown rat	▲	▲	▲	▲	▲	▲ (1:30)
8	Brown rat	–	–	▲ (1:300)	▲ (1:300)	–	–
9	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
10	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
11	Brown rat	▲	▲	▲	▲ (1:30)	▲	▲
12	Brown rat	▲ (1:30)	▲	▲	▲ (1:1,000)	▲	▲
13	Brown rat	▲	▲	▲	▲ (1:30)	▲	▲
14	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
15	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
16	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
17	Brown rat	–	–	▲ (1:1,000)	▲ (1:300)	–	▲ (1:300)
18	Brown rat	–	–	▲ (1:100)	▲ (1:300)	–	▲ (1:30)
19	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
20	Brown rat	▲	▲	▲	▲ (1:100)	▲	▲
21	Brown rat	▲	–	▲ (1:30)	▲ (1:100)	–	▲ (1:100)
22	Brown rat	▲	▲ (1:100)	▲	▲ (1:100)	▲	▲ (1:100)
23	Brown rat	▲	▲	▲ (1:30)	▲	▲	▲
24	Brown rat	▲	▲ (1:30)	▲ (1:30)	▲	▲	▲
25	Brown rat	▲	▲	▲ (1:30)	▲	▲	▲
26	Brown rat	▲	▲	▲ (1:30)	▲	▲	▲

**Leptospira* spp. DNA was detected by real time PCR (qPCR) in the kidneys of this specimen

of rodents in the epidemiological cycle of this bacteria in anthropized areas (Adler and de la Peña Moctezuma, 2010; Boey et al., 2019). All *Leptospira* serovars agglutinated by MAT in the brown rats analyzed (Copenhageni, Grippotyphosa, Pomona, Canicola and Hardjo) have also been previously described in wild rodents in urban and periurban environments (Bharti et al., 2003; Boey et al., 2019; Guglielmini et al., 2019). Our results suggest circulation of different serovars in the brown rat population of the CZCC, however, cross-reactions among serovars cannot be ruled out.

5. Conclusion

Our results confirmed *L. interrogans* infection as the cause of sudden mortality in captive Barbary macaques in Spain, with sympatric wild rodents being the most likely reservoir involved in transmission. The present study provides evidence of the high susceptibility of this endangered NHP species to *Leptospira* spp. infection. Based on our results, the implementation of rodent control programs could be an effective measure to minimize the exposure of susceptible species to *Leptospira* spp. in zoos. Given the importance of leptospirosis for animal and public health, as well as for conservation, surveillance programs for *Leptospira* spp. should be implemented in both captive species and free-living sympatric rodents in zoos worldwide.

Ethical approval

All animals and samples used in this study were referred by zoo staff to the University of Cordoba following an outbreak of disease. No ethical approval was necessary.

CRediT authorship contribution statement

Beato-Benítez Adrián: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cano-Terriza David:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Mozos-Mora Elena:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Investigation. **Guerra Rafael:** Writing – review & editing, Validation, Supervision, Resources, Investigation. **García-Bocanegra Ignacio:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Martínez Remigio:** Writing – review & editing, Validation, Supervision, Methodology. **Gonzálvez Moisés:** Writing – review & editing, Supervision, Software, Formal analysis, Data curation. **Ruano María José:** Writing – review & editing, Validation, Methodology, Data curation. **Pérez-Cobo Iratxe:** Writing – review & editing, Validation, Methodology, Data curation.

Declaration of Competing Interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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