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Monitoring of pathogenic *Leptospira* infection in wolves (*Canis lupus*) from Spain and Italy

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ABSTRACT

Leptospirosis is a bacterial disease of worldwide distribution with relevant implications for animal and human health. Different large wild carnivore species can act as reservoirs of this zoonotic pathogen. This study aimed to evaluate the circulation of *Leptospira* spp. in free-ranging wolves (*Canis lupus*) from southern Europe. A total of 281 kidney samples of wolves from Spain and Italy were collected between 2017 and 2023. The presence of *Leptospira* DNA was analysed by real-time PCR and phylogenetic analyses were carried out using a Bayesian approach. The overall prevalence was 3.2 % (9/281; 95 %CI: 1.1–5.3). *Leptospira* DNA was detected in nine of the 180 wolves from Spain (5.0 %; 95 %CI: 1.8–8.2), but not in the Italian wolf population (0 %; 0/101). Molecular analyses revealed high homology between the sequences obtained in the present study and isolates of *Leptospira interrogans* and *Leptospira borgpetersenii* from different rodent and domestic ungulate species. Our results provide evidence of a low and spatially heterogeneous circulation of this pathogen in wolf populations of southern Europe. The detection of zoonotic *Leptospira* species in this survey supports the need to consider wolf populations in monitoring programs for leptospirosis with a One Health approach.

1. Introduction

Leptospirosis is a bacterial zoonotic disease affecting more than 160 mammal species worldwide. The infection by pathogenic *Leptospira* (order Spirochaetales, family Leptospiraceae) annually causes more than 60,000 human deaths and has important health, economic and conservation implications for domestic and wild animals (Adler and de la Peña Moctezuma, 2010). Several routes of *Leptospira* spp. transmission have been reported, although direct contact with leptospiral-contaminated water, soil or secretions (e.g., blood, urine…), as well as trophic interactions with infected individuals stand out (Bharti et al., 2003; Adler and de la Peña Moctezuma, 2010; Bradley and

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Lockaby, 2023).

Among the wide range of competent reservoirs of *Leptospira* species, wild carnivores can act as key hosts promoting the maintenance and transmission of this pathogen in natural and anthropized environments (Bharti et al., 2003; Vieira et al., 2018). In this sense, the recent geographical expansion of some large carnivore species in Europe (e.g., lynxes, bears, wolves…) (Chaprón et al., 2014; Blanco and Sundseth, 2023) may increase the likelihood of interspecific interactions, thereby fostering the spread of multi-host pathogens affecting both animals and humans (Wymazał et al., 2024). In fact, changes in pathogen dynamics have been reported in wolf populations recolonizing their historic ranges in some European regions (Lesniak et al., 2017, 2018). In Europe, management and conservation strategies focused on the preservation of wolves, which was close to the extinction in the 20th century, have been actively implemented during the last decades. Specifically, conservation programs and national regulations for restoring the natural distribution of Italian (*Canis lupus italicus*) and Iberian (*Canis lupus signatus*) wolf populations (e.g., LIFE12 NAT/IT/000807; LIFE15 GIE/ES/000962; TED/980/2021) have been implemented, resulting in the establishment of solid wolf packs (23.2–30.0 % wolves from the European Union; Blanco and Sundseth, 2023).

Although the exposure of wolves to pathogenic *Leptospira* species has been previously reported in Europe (Millán et al., 2014; Bregoli et al., 2021; Žele-Vengušt et al., 2021), molecular studies addressing the active circulation of this pathogen are still very limited. Consequently, our study aimed to evaluate the prevalence of *Leptospira* spp. in free-ranging wolf populations inhabiting the southern Europe.

2. Material and methods

2.1. Study design and sample collection

A total of 281 free-ranging wolves, which were road-killed or legally hunted, were sampled in three provinces from the northern Spain (n = 180) and ten provinces from the northwestern Italy (n = 101) between 2017 and 2023. The sampling area in northern Spain is mainly characterized by the presence of broad-leaved and mixed forests with an altitude varying from 400 to 2600 m above sea level (m.a.s.l.) predominating mild winters (6ºC-13ºC) and moderate summers (17ºC-24ºC). The northwestern Italy is mainly characterized by a mountainous ecosystem (Italian Alps) composed of coniferous and mixed forests with an altitude ranging from 350 to 4800 m.a.s.l., in which cold winters (from −5ºC to 4ºC) and mild summers (15ºC-24ºC) on valley floors stand out. All animals were carefully necropsied by trained veterinarians to determine the cause of death, and samples of kidneys were individually collected in sterile bags and stored at −20ºC until laboratorial analyses. Epidemiological information from sampled individuals, including animal origin (province), sampling date, sex and age was recorded whenever possible. Age of wolves was categorized according to the tooth wear and body size, as follow: juveniles (<2 years), subadults (2–3 years) and adults (>3 years) (Brasington et al., 2023).

2.2. Molecular analyses

For the molecular detection of *Leptospira* spp., total genomic DNA was extracted from approximately 25 mg of kidney samples using the NucleoSpin® Tissue kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions and stored at –20°C until analysis. A fragment of 242 bp from the *lipL32* gene, present only in pathogenic leptospires, was amplified by real-time PCR (qPCR) using the primers LipL32–45 F (5′-AAGCATTACCGCTTGTGGTG-3′) and LipL32–286R (5′-GAACTC CCATTTCAGCGATT-3′), and the TaqMan probe LipL32–189 P (5′- FAM-5′-AAAGCCAGGACAAGCGCCG-3′-BHQ1–3′), as previously described (Stoddard et al. 2009). The qPCR was carried out in the CFX96 real-time PCR detection system (Bio-Rad).

Subsequently, the positive qPCR samples were analysed with conventional nested PCR (cPCR) using the primers secYF (5′-ATGCC-GATCATTTTTGCTTC-3′) and secYR (5′-CCGTCCCTTAATTTTA-GACTTCTTC-3′), and nested primers secYIVF (5'-GCGATTCAGTTTAATCCTGC-3') and secYIV (5'-CTTA-GATTTGAGCTCTAACTC-3') that amplifies a 549 and 201 bp fragment of the *secY* housekeeping gene of *Leptospira* spp., respectively (Ahmed et al., 2006, 2009). Conventional PCR products were visualized by electrophoresis in 1.5 % agarose gels. DNA bands from the amplified cPCR products were purified using the QIAquick PCR and Gel Cleanup Kit (QIAGEN®, Hildem, Germany) in accordance with the manufacturer's instructions and directly sequenced by StabVida (Caparica, Portugal) using the nested primers employed for the DNA amplification. The nucleotide sequences obtained were assembled and edited using the software ChromasPro and then the consensus sequences were compared for similarity with other available sequences in the GenBank using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). After that, an alignment was carried out using the online tool Multiple Sequence Alignment by CLUSTALW (https://www.genome.jp/tools-bin/clustalw). Afterwards, a phylogenetic analysis was carried out through a Bayesian approach using the software MrBayes 3.2.7 (Ronquist et al., 2012). The phylogenetic tree was constructed based on the Hasegawa-Kishino-Yano model with gamma-distributed rate variation across sites (HKY+G) (Hasegawa et al., 1985), as estimated by the Akaike Information Criterion (AIC) using the software jModelTest (Darriba et al., 2012). To construct the tree, a total of 51 nucleotide sequences were used, including sequences obtained in this study as well as relevant *Leptospira* species available from GenBank. Additionally, a sequence of *Leptospira biflexa* (Accession number: NC_010602.1) was included as outgroup. The sampling was carried out by Markov Chain Monte Carlo (10,000,000 generations sampling every 1000 steps). The obtained phylogenetic tree was visualised and edited with the software FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 1.3 (https://inkscape.org/).

2.3. Statistical analyses

The prevalence of pathogenic leptospires infection was estimated as the quotient between the number of positive animals and the total number of animals evaluated with a 95 % confidence interval (95 %CI) (Thrusfield and Christley, 2018). Bivariate associations between the prevalence to *Leptospira* spp. and explanatory variables were analysed using the Pearson's chi-squared test or Fisher's exact test, as appropriate. All statistical analyses were performed using R software version 4.1.3 and significant differences were considered with $P \leq 0.05$ for a double-sided test.

3. Results and discussion

Leptospira DNA was detected in 9 of the 281 wolves analysed (3.2 %; 95 %CI: 1.1–5.3). To the best of the author's knowledge, this is the largest epidemiological survey evaluating circulation of *Leptospira* species in wolves so far. Our results provide a broader viewpoint about *Leptospira* infections in this large carnivore, since previous surveys carried out in this species are scarce and geographically restrained (Millán et al., 2014; Bregoli et al., 2021; Mazzotta et al., 2023). The overall individual prevalence detected in the present study indicates a low active circulation of pathogenic leptospires in the wolf populations analysed.

Statistically significant differences between countries were found (P = 0.028), detecting *Leptospira*-positive wolves in Spain (5.0 %; 9/180; 95 %CI: 1.8–8.2), but not in the Italian wolf populations (0 %; 0/101) analysed (Fig. 1). At least one positive individual was detected in the three Spanish provinces sampled with prevalence values ranging between 4.2 % and 10.0 % (Fig. 1). The prevalence obtained in Spain is lower than the 16.3 % (8/49) previously observed in wolf populations inhabiting the northern of the Iberian Peninsula (Millán et al., 2014).

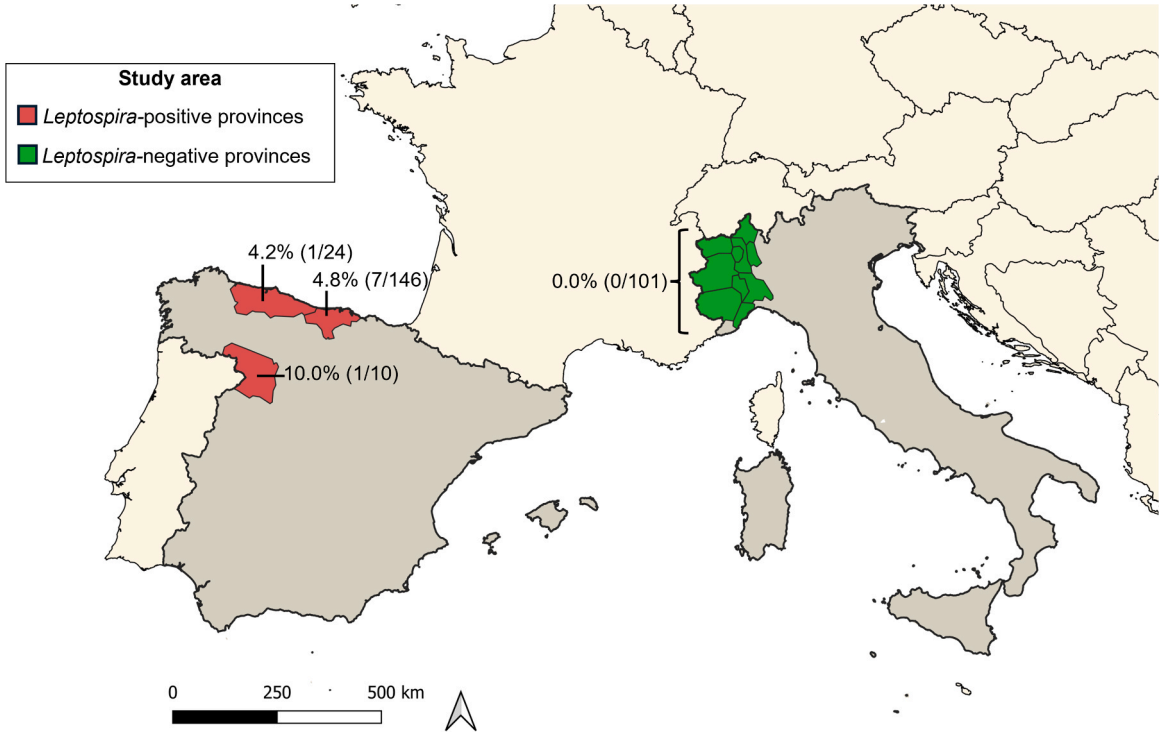

Fig. 1. Geographical distribution of provinces from Spain and Italy where wolf samples have been collected. Prevalence (*Leptospira*-positive wolves/total individuals) in each Spanish province sampled is shown.

In Spain, statistically significant differences between positivity and sex or age classes were not found. Although the low prevalence detected in the present study could limit the statistical significance, a trend in *Leptospira* prevalence was observed, with a higher number of positive animals found as their age increased. *Leptospira* DNA was detected in adult (55.6 %; 5/9; 95 %CI: 23.1–88.1) and subadult individuals (22.2 %; 2/9; 95 %CI: 5.0–49.4), but not in juveniles (0.0 %; 0/45) ($P = 0.406$). Our results are in line with findings observed by Millán et al. (2014), evidencing a higher *Leptospira* infection in older wolves. This issue is consistent with chronic infections of *Leptospira* spp. in domestic dogs, where excretion of this spirochete has been detected even several years after infection (Mauro and Harkin, 2019; Hetrick et al., 2022). At least one *Leptospira*-positive wolf was detected in four (2017, 2018, 2019 and 2021) of the seven sampling years, which is in concordance with those reported in the same study area between 2010 and 2013 (Millán et al., 2014). These findings indicate an endemic circulation of *Leptospira* species in the wolf populations from this region. During the *postmortem* examination, none of the *Leptospira*-positive wolves presented macroscopic lesions compatible with leptospirosis, which suggest the role of this carnivore species as asymptomatic reservoir of *Leptospira* spp.

Regarding Italian wolves, *Leptospira* DNA was not found in the 101 kidneys analysed. In northeastern Italy, Mazzotta et al. (2023) detected *Leptospira* spp. in one of the 13 (7.7 %) wolves sampled during 2015–2022. Bregoli et al. (2021) also reported infection by this pathogen in a road-killed wolf in 2018 in the northeastern Italy. Previous studies have evidenced antibodies to *L. interrogans* in wolves from central and northern Europe, as have been reported in Norway/Sweden (8.4 %; 8/95) and Slovenia (66.7 %; 2/3) (Akerstedt et al., 2010; Žele-Vengušt et al., 2021). Similarly, *Leptospira* spp. circulation has also been detected in serosurveys performed in wolves from North America, particularly in regions of United States, such as Minnesota (11.4 %; 52/457) and Alaska (1.0 %; 1/82) (Zarnke and Ballard, 1987; Khan et al., 1991).

Seven of the nine qPCR-positive samples from Spanish wolves amplified using the cPCR. After purification, these samples were sequenced and registered in the GenBank database with the accession numbers PP372553-PP372559. The obtained sequences of the partial *secY* gene of *L. interrogans* ($n = 3$) showed a percentage of similarity between 99.8 % and 100 % with the sequences obtained from pigs (*Sus scrofa domestica*) and humans (Fig. 2). By contrast, the obtained sequences of *L. borgpetersenii* ($n = 4$) showed a percentage of similarity between 99.8 % and 100 % with other sequences obtained from rodents (*Rattus* spp., *Mus* spp. and *Apodemus* spp.) and humans (Fig. 2). *Leptospira interrogans* has been previously reported in wolves and other wild carnivore species in the study area (Millán et al., 2014; Mazzotta et al., 2023). Although *L. borgpetersenii* has been isolated in some free-ranging carnivores in the Iberian Peninsula (Millán et al., 2009), this is the first report of this *Leptospira* species in wolves, which increases the range of hosts susceptible to this pathogen. Interestingly, the high homology between our sequences and those detected in different rodent and domestic ungulate species might indicate the infection source of pathogenic leptospires in wolves inhabiting Iberian ecosystems. In this sense, trophic interactions between wolves and other sympatric mammals (e.g., rodents, wild ungulates), which usually act as predilect prey of this top predator, may be the most plausible transmission pathway for *Leptospira* infection (Ferretti et al., 2019; Janeiro-Otero et al., 2022). However, not only predator-prey interactions, but also consumption of ungulate carrion or exposure to contaminated water, soil or secretions have been suggested to be source of *Leptospira* infection in wolves (Adler and de la Peña Moctezuma, 2010; Espí et al., 2000, 2010; Millán et al., 2014; Bradley and Lockaby, 2023).

As future perspective, the current eco-epidemiological scenario of pathogens for which wolves may act as natural reservoirs could change during the next few years due to the geographical expansion of this carnivore species in Europe (Chaprón et al., 2014; Pettersson et al., 2021). Also, the expansionist trend of wolf populations colonizing anthropized areas may favour the existence of a close wildlife-domestic-human interface allowing the circulation of shared pathogens, including *Leptospira* species (Pettersson et al., 2021). In this sense, the northern Spain is considered a hotspot area for *Leptospira* spp. circulation among wildlife, domestic animals and humans (Espí et al., 2000, 2010; Millán et al., 2019; López et al., 2019; RENAVE. 2024). The

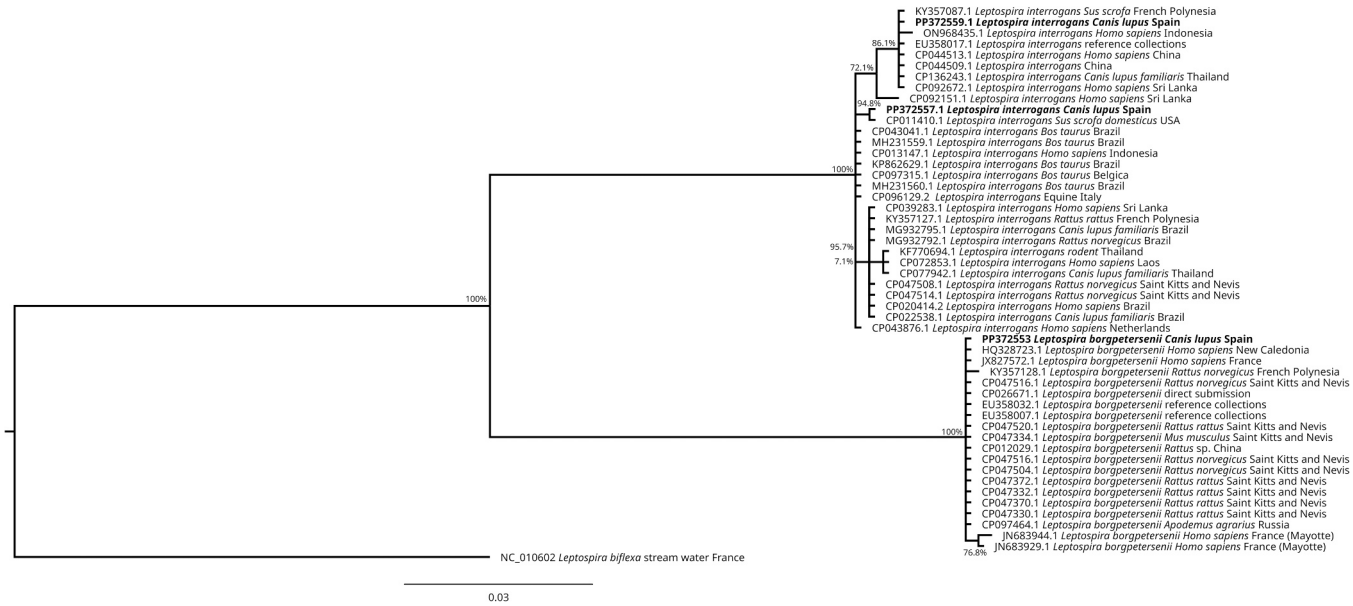

Fig. 2. Phylogenetic tree of the partial *secY* gene of *Leptospira* spp. The tree was obtained using a Hasegawa-Kishino-Yano model with gamma-distributed rate variation across sites (HKY+G) with MrBayes software 3.2.7 (Ronquist et al., 2012), using Bayesian inference with Markov Chain Monte Carlo sampling (10,000,000 generations, sampling every 1000 generations). This analysis involved 51 nucleotide sequences. The nucleotide sequence of *Leptospira biflexa* was used as an outgroup. Isolates obtained in this study are highlighted in bold.

relevance of animals as reservoirs of *Leptospira* spp. requires the evaluation of this zoonosis under a One Health perspective, in which the monitoring of potential wild and domestic hosts becomes pivotal to prevent human leptospirosis (Bharti et al., 2003; Adler and de la Peña Moctezuma, 2010). Our results point the need to continue studying the complex network of wild hosts involved in the epidemiological cycle of *Leptospira* spp. in Europe. In this sense, further serosurveys evaluating the exposure of wolves to *Leptospira* spp. in our study area are encouraged.

In conclusion, the present large-scale study evidences a low, endemic and heterogeneous circulation of pathogenic leptospires in wolf populations from the southern Europe. The detection of zoonotic *L. interrogans* and *L. borgpetersenii* in the Spanish wolf populations is of public health concern and support the need to consider this large carnivore in monitoring programs of *Leptospira* spp. from a One Health approach.

CRediT authorship contribution statement

Roser Velarde: Writing – review & editing, Visualization, Supervision. **Clara Muñoz-Hernández:** Writing – review & editing, Resources, Methodology. **Riccardo Orusa:** Writing – review & editing, Validation, Resources, Funding acquisition. **Susana Remesar:** Writing – review & editing, Methodology, Investigation. **David Cano-Terriza:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Ignacio García-Bocanegra:** Writing – review & editing, Visualization, Project administration, Funding acquisition, Conceptualization. **Moisés Gonzálvez:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Manena Fayos:** Writing – review & editing, Resources, Data curation. **Álvaro Oleaga:** Writing – review & editing, Validation, Resources, Methodology. **Serena Robetto:** Writing – review & editing, Methodology, Investigation, Data curation. **Remigio Martínez:** Writing – review & editing, Visualization, Methodology, Investigation. **Barbara Moroni:** Writing – review & editing, Resources, 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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