Wildlife as a sentinel for pathogen introduction in non-endemic areas: Is *Leishmania tropica* circulating in wildlife in Spain?

Authors: Iris Azami-Conesa[1,2]; Pablo Matas Méndez[3]; Paula Pérez-Moreno[1]; Javier Carrión[1], J.M. Alunda[1], Marta Mateo Barrientos*[4]; María Teresa Gómez-Muñoz*[1]

Affiliations

1. Department of Animal Health, Faculty of Veterinary Sciences, University Complutense of Madrid, Madrid, Spain
2. Universidad Europea de Madrid. Faculty of Biomedical and Health Sciences. Department of Pharmacy and Nutrition, Villaviciosa de Odón, Madrid, Spain.
3. Faculty of Veterinary Sciences, University Alfonso X el Sabio, Villanueva de la Cañada, Madrid, Spain.
4. Department of Microbiology and Parasitology, Faculty of Pharmacy, University Complutense of Madrid, Madrid, Spain.

* These authors contributed equally to this work and share last authorship.

Correspondence: María Teresa Gómez Muñoz (mariateg@ucm.es) and Marta Mateo Barrientos (mmateo14@ucm.es).

Keywords: Wildcat, *Leishmania tropica*, Spain, First description, autochthonous; Iberian Peninsula; PCR; genotyping.

Abstract

Background

Leishmaniasis is a global arthropod-borne zoonotic disease of chronic course and cutaneous, mucocutaneous, and visceral clinical manifestations. In the Iberian Peninsula, only *Leishmania infantum* has been reported, although other species of *Leishmania*, such as *L. tropica* and *L. major*, are present in surrounding countries.

Aim

The aim of this work is to analyse the occurrence of *Leishmania* spp. infection in wildcats as sentinels, including their genotypic characterization.

Methods

Necropsies of 18 roadkill wildcats were carried out. Samples from ear skin and spleen were taken, and DNA isolation and PCR analysis of the highly sensitive *SSUrDNA* target were conducted. Subsequent PCR tests were conducted using more specific targets for *Leishmania* species determination: *hsp70* and *ITS1*Positive samples were sequenced, and phylogenetic trees were constructed.

Results

Seven out of 18 wildcats were found positive to *Leishmania* spp. in ear skin; of them, only two were positive in spleen samples. . Based on the *hsp70* and *ITS1* sequences, one animal was found to be infected solely with *L. tropica* in ear skin samples, while two cats were found to be infected with *L. infantum* in both ear and spleen. Another animal was found to be co-infected with *L.*

infantum and *L. tropica* in ear skin. The *Leishmania* species could not be determined in three cats as the targeted *SSUrDNA* is only specific to the genus.

Conclusions

This is the first report of autochthonous *L. tropica* infection in the Iberian Peninsula. Health care professionals, including physicians, dermatologist and veterinarians must be aware of this for a correct diagnosis, treatment, and management of possible co-infections.

Introduction

Leishmaniasis is a global zoonotic disease transmitted by the bite of phlebotomine insects, with more than 1,000,000 new cases diagnosed annually in 92 endemic countries [(1)]. Clinical presentation in humans is variable and includes cutaneous (CL), mucocutaneous (MCL), visceral (VL), and Post Kala-azar dermal leishmaniasis (PKDL). The disease is caused by several species of *Leishmania*, with *L. donovani* complex, *L. major* and *L. tropica* complex being the most frequent in Africa, Asia, southern Europe, South America, and the Middle East, while *L. mexicana* complex, *L. guyanensis* complex and *L. braziliensis* complex are the most diagnosed in the Americas. The cutaneous form of the disease is the most common and is responsible of the stigmatization and social isolation of thousands of people, particularly women, while VL is usually lethal without treatment and is caused by *L. donovani* in Africa and Europe, and *L. infantum* in southern Europe and the Americas. MCL is characterized by the destruction of mucous tissue of the nose, palate, and pharynx, and can severely affect the daily life of thousands of people [(2,3)]. Human leishmaniasis is more prevalent in low-income countries with displaced populations due to war, social or economic conflicts and is classified by WHO as a neglected tropical disease (NTD). This scenario makes early diagnosis and treatment of the disease, as well as the introduction of preventive measures, difficult (3)].

In Europe, VL is caused by *L. infantum* with both humans and dogs as target species. The infection is more prevalent in southern countries, such as Portugal, Spain, Italy, and Greece. Dogs are heavily affected by this zoonotic species [(4)] and are considered the main reservoir. Human infections mainly affect immunocompromised patients (such as those HIV+ or recipients of solid organs transplants) as well as occasional reports in elderly and children [(5)]. Several studies have stressed the paramount importance of wildlife in maintaining *Leishmania* infection [6,7]. Wildcats (*Felis silvestris*) are susceptible to the same pathogens as domestic cats and therefore could be potential reservoirs for the infection. However, studies conducted have been scarce [8–10], with a limited number of animals analysed in each study (n=4, n=3, n=4, respectively). The three studies identified *L. infantum* using a small fragment of kDNA or qPCR of the ITS2 regions.

Despite suitable bioclimatic conditions and available vectors for the anthroponotic *L. tropica* (*Phlebotomus sergenti*) and *L. major* (*P. papatasi*) [11,12], only *L. infantum* has been reported in the Iberian Peninsula [6,12,13]. However, *L. major* has been recently reported in a cat from Lisbon, Portugal [14]. Moreover, it is worth noting that *L. tropica* has been previously reported in Greece [15] and the three species (*L. infantum, L. tropica* and *L. major*) are present in Morocco [16], as well as in other countries bordering Europe [17]. Availability of a larger number of wildcats allowed us to identify the presence of *Leishmania* in various tissues and genetically characterize the samples.

Material and methods

Animals, samples, and geographic area

Samples from 18 wild cats that were road killed during 2022 and 2023 in the Spanish provinces of Burgos, Valladolid, Soria, Ávila, Guadalajara, and Ciudad Real were analysed in this study.

The animals were transported to the wildlife recovery centres of Castilla y León and Castilla-La Mancha, systematic necropsies were performed, and the presence/absence of lesions annotated. A portion of the ear skin and the spleen were taken and frozen at -20°C for further processing. Date, age, sex, and locality (geographic coordinates where the dead animals were found) were included in the data base.

Maps

The map was created by taking as reference the maps of distribution in Europe of *Phlebotomus sergenti*, the main vector of *L. tropica*, in 2022 and 2023 [18], and the geographic locations of the wildcats analysed in this study. The AutoCAD program (Autodesk® Inc., the Landmark, San Francisco, CA, USA) was employed to observe the overlaps between the two maps.

DNA isolation

DNA was isolated from spleen (10 mg) and ear skin (20 mg) using the NZYTech Tissue gDNA isolation kit (NZYTech, Portugal) and following the manufacturer's instructions . DNA elution was conducted in 60 µL of elution buffer instead of 100 µL to increase DNA concentration. Positive and negative controls were used in each batch of the experiment. The positive control consisted of samples derived from *L. major* (clone V1: MHOM/IL/80/Friedlin) or *L. braziliensis* (MHOM/BR/75/M2904) promastigotes in culture ($1x10^6$ promastigotes), while the negative control consisted of DNase-free water. DNA content was measured in a Multiskan™ GO microplate spectrophotometer with µDrop™ plates (Thermo Fisher Scientific, MA, USA). DNA from cultures of *L. major* and *L. braziliensis* were obtained and used as internal controls for PCRs and sequencing.

PCR of Small subunit ribosomal RNA (SSU-rDNA), Internal Transcribed spacers-5.8 ribosomal DNA 1 (ITS1), and heat shock protein 70 (hsp70) targets

DNA samples from ear skin and spleen of the 18 cats were used for *Leishmania* DNA detection. DNA from different *Leishmania* species was also used as an internal control for PCR and sequencing: *L. major* (clone V1: MHOM/IL/80/Friedlin) and *L. braziliensis* (MHOM/BR/75/M2904). Only samples with good DNA quality (absorbance ratio at 260/280 nm > 1.7) were used for all PCRs. All methods have been previously described and adapted to the working conditions recommended by the enzyme manufacturer (Supreme NZYTaq II 2x Master Mix, NZYTech, Lisbon, Portugal).

Nested PCRs (nPCR) to amplify a 358 bp fragment of the *SSU-rDNA* target were performed using primers R221 (5´- GGTTCCTTTCCTGATTTACG-3´) and R332 (5´- GGCCGGTAAAGGCCGAATAG-3´) for the outer section, and primers R223 (5´- TCCATCGCAACCTCGGTT-3´) and R333 (5´- AAAGCGGGCGCGGTGCTG-3´) for the inner section [19], following previously described conditions [20].

Primers L5.8S (5´- ACACTCAGGTCTGTAAAC-3´) and LITSR (5´- CTGGATCATTTTCCGATG-3´) were used for the outer section of the *ITS1* region and primers SAC (5´- CATTTTCCGATGATTACACC-3)´and VAN2 (5´- CGTTCTTCAACGAAATAGG -3´) were used for the inner section to amplify a fragment of 280-330 bp, as previously described [20,21].

The nPCR used for *hsp70* amplified a fragment of 741 bp and was based on the primers and methods previously described for phylogenetic purposes [14,22]. Briefly, external primers HSP70-F25 (5´-GGACGCCGGCACGATTKCT-3´) and HSP70-R1310 (5´-CCTGGTTGTTGTTCAGCCACTC-3´) were used at 0.8 µM each , and internal primers HSPF (5´-GACAACCGCCTCGTCACGTTC-3´) and HSPR (5´-GTCGAACGTCACCTCGATCTGC-3´) were used at 0.4 µM each. In the first PCR, 40 cycles of 94°C for 40 s, 61°C for 60 s and 72°C for 120 s were used, while in the second PCR, 40 cycles of 94°C for 40 s, 61°C for 60 s and 72°C for 60 s were used.

All PCR reactions were performed in 25 µL using 12.5 µL of Supreme NZYTaq II 2x Master Mix. An initial step of 95°C for 5 min to activate the enzyme, and a final elongation step of 72°C for 10 min to allow the elongation of PCR products were employed in all PCR reactions. In the first PCR, 5 µL of DNA was used, while in the second PCR reactions 5 µL of a 1:50 dilution from each first PCR product were employed for *hsp70*, while a 1:40 dilution of the first PCR product was used for SSU-rRNA and ITS1.

The results of the nPCR were visualized in 1% agarose gels stained with SYBR™ safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, MA, USA) under UV light.

Sequencing and alignment

Samples containing the amplicons of the expected size were sent to the facilities of Macrogen Spain (Madrid, Spain)for Sanger sequencing. Samples were purified and sequenced using a DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) and the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), following the manufacturer's instructions. The resulting sequences were aligned using Molecular Evolutionary Genetics Analysis v.11 software (MEGA XI) [(23)] and FinchTV 1.4.0 software (Geospiza, Inc.; Seattle, WA, USA), manually checked, and subjected to BLAST analysis using the nucleotide Basic Local Alignment Search Tool (BLAST) (National Library of Medicine, Rockville, MD, USA).

Only sequences with more than 150 bp that were clear in both directions were sent to GenBank for accession numbers.

Phylogenetic trees

Phylogenetic trees were constructed using the sequences obtained in the present study and other sequences available at GenBank. Trees for *hsp70* and *ITS1* are shown because *SSU-rDNA* target is a conserved region and only a difference of 1-2 bp was observed between the sequences obtained.

In all trees, a sequence from *Trypanosoma cruzi* was included as an out-group reference. In addition, other sequences of *Leishmania* spp. from GenBank and sequences from *L. infantum* and the above-mentioned species of *Leishmania* from cultures were included to verify similarity (*L. major,* and *L. braziliensis*). Sequences had a minimum length of 236 nucleotides for *ITS1* region and 635 nucleotides for *hsp70*. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura–Nei model [24]. The tree with the highest log likelihood was displayed. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Neighbour-Joining and BioNJ algorithms to a pairwise distance matrix estimated using the Maximum Composite Likelihood (MCL) approach and selecting the topology with the superior log likelihood value. Trees were drawn to scale, with branch lengths measured based on the number of substitutions per site. The analysis included 13-18 nucleotide sequences,

depending on the tree. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted via MEGA XI [23]. A bootstrap of 2000 replicates was used to assess the reliability of the trees.

Statistical analysis

A descriptive statistical analysis with absolute (n) and relative frequency of infection (%) was carried out, with a confidence level of 95%, using the free online tool WinEpi (Working in Epidemiology) and considering that the diagnostic techniques employed were perfect (nPCR of the *SSUrDNA*). Odds ratio analysis was used to analyse the risk factors "sex" and "*Leishmania* infection" [25].

Results

Detection and identification of Leishmania *spp. in wild cat*

Samples from ear skin and spleen of eighteen wildcats have been analysed in this study, except for the spleen of two animals that were in bad condition when the cats were found (Table 1). None of the animals displayed lesions comparable to those produced by *Leishmania* infections. Considering all the animals positive for *Leishmania* PCR, 38.89% (16.37-61.41%, 95% Confidence interval) were found to be infected. A non-significant higher proportion of infection was found in males (OR= 27.17, 95% CI 0.33, 2213.63), since only males were found infected, but we must consider that we obtained a smaller number of samples from females (n=3/18).

Table 1. Age, sex, geographical location, date of collection and *Leishmania* infection of the studied wildcats.

	Age	Sex	Date	Location	*Leishmania* infection
262	Adult	M	20-04-2022	Villagonzalo Pedernales (Burgos)	+
263	Adult	M	21-04-2022	Bonilla de la Sierra (Ávila)	+
268	Adult	M	22-06-2022	Sedano (Burgos)	+
272	Adult	M	13-07-2022	Cogeces de Íscar (Valladolid)	-
275	Adult	M	15-07-2022	Berlangas de Roa (Burgos)	+
276	Adult	M	15-07-2022	Torreblancos (Soria)	-
310	Adult	M	21-12-2022	Almazán (Soria)	-
317	Adult	M	24-02-2023	Temiño (Burgos)	-
318 *	Adult	M	13-03-2023	Castrillo de Riopisuerga (Burgos)	+
319	Adult	F	13-03-2023	Hermosilla (Burgos)	-
320	Adult	M	23-03-2023	Cubo de la Solana (Soria)	-
323	Adult	M	28-04-2023	Adradas (Soria)	+
365	Adult	M	08-09-2023	Cascajares de Bureba (Burgos)	+
366	Adult	M	08-09-2023	Martialay (Soria)	-
367	Adult	F	08-09-2023	Retuerta (Burgos)	-
375	Adult	M	05-10-2023	Fuencaliente (Ciudad Real)	-
376	Adult	F	05-10-2023	Pedrosa del Príncipe (Burgos)	-
378 *	Adult	nd	05-10-2023	Peralejo (Guadalajara)	-

*Spleen not analyzed. M: male; F: female. nd: not determined.

Seven of the eighteen animals were found positive by *Leishmania* PCR for SSU-rDNA. All were positive at the ear skin, and only two were also positive in spleen samples (Table 2).

Table 2. Results of end-point PCR using samples from ear skin and spleen and three targets for *Leishmania* DNA amplification. Sequences identities are identified with super index numbers (1-4).

Sample	Ear skin			Spleen		
	SSU-rDNA	*ITS1*	*hsp70*	*SSU-rDNA*	*ITS1*	*hsp70*
262	+	-	-	-	-	-
263	+	+[2]	+[4]	+	+[2]	+[4]
268	+	+[2]	+[3]	-	-	-
275	+	+[1]	+[3]	-	-	-
318	+	-	-	nd	nd	nd
323	+	+[2]	+[4]	+	+[2]	+[4]
365	+	-	-	-	-	-

nd: not determined. 1: *ITS1* sequence 100% identity with *L. tropica*; 2: *ITS1* sequence 100% identity with *L. infantum*; 3: *hsp70* sequence 99.86-100% identity with *L. tropica*; 4: *hsp70* sequence 99.24-100% identity with *L. infantum.*

Sequences and GenBank accession numbers

Nucleotide sequences from wildcats obtained in this study were deposited in the GenBank database under accession numbers PP389513-PP389521 for *SSU-rDNA*, PP177368-PP177373 for *ITS1*, and PP397157-PP397162 for *hsp70*. Sequences from *L. major* and *L. braziliensis* isolates were deposited in the GenBank database with accession numbers PP389522, PP389523, and PP389533.

Sequences obtained from *SSU-rDNA* had 295-327 bp and differ by only 1 or 2 nucleotide positions. Nine sequences were obtained from the analysed animals, seven from the ear skin and two from the spleen. Seven of the obtained sequences displayed 100% query coverage and 99.69-100% identity with other *L. infantum* sequences available at GenBank (acc. n MN757921, obtained from *Canis lupus*, Brazil). Sequences obtained from the ear skin of animals 365 and 323 showed 100% query coverage and 99.69-100% identity, respectively, with sequences from *L. major*, *L. enrietti, L. infantum, L. mexicana, L. guyanensis* or *L. tropica* (acc. n MT560279, MK737948, MK495994, KM061042, X53913, KF302745), being identical except one nucleotide.

ITS1 sequences of 236-247 bp were obtained from the ear skin of four animals (263, 268, 275, 323), two of which (263 and 323) also gave sequences when DNA from the spleen was used (Table 2). Three sequences displayed 100% query coverage and 100% identity with sequences from *L. infantum* (acc. n MT416168, samples from ear skin of animals 263, 268, and 323, and samples from spleen of animals 263 and 323), while one sequence (from ear skin of wildcat 275) displayed 100% query coverage and 100% identity with sequences from *L. tropica* (acc. n MH595857, isolate R24, Iran).

Sequences obtained from *hsp70* amplification yielded four sequences from the ear skin and two sequences from the spleen with 635-711 bp. Of these, four sequences showed 100% coverage and 99.24-100% identity with *L. infantum* (acc. n OR136937, strain MHOM/IT/99/ISS1898). The other two sequences (268 and 275 from ear skin) displayed 100% coverage and 99.86 and 100% identity, respectively, with *L. tropica* (acc. n LN907846, strain MHOM/IL/80/SINGER, and MK335938, voucher ISS3183) (Table 2). Double peaks were observed in three and five positions of sequences from the spleen of animal 323 and ear skin of animal 263, respectively, indicating mixed sequences.

Considering all sequences obtained with *SSUrDNA*, *ITS1* and *hsp70* targets, *L. tropica* tends to be better amplified in samples from ear skin than in samples from spleen. In one animal (275) sequences from the *ITS1* and *hsp70* targets showed 100% identity with *L. tropica*. Two animals displayed sequences compatible with *L. infantum* at ear skin and spleen (263 and 323), and another wildcat was positive only at ear skin with sequences compatible with *L. infantum* and *L. tropica* (268). Finally, in three animals (262, 318 and 365) the sequences could only by assigned to *Leishmania* genus because they were only positive for *SSUrDNA* (Table 2).

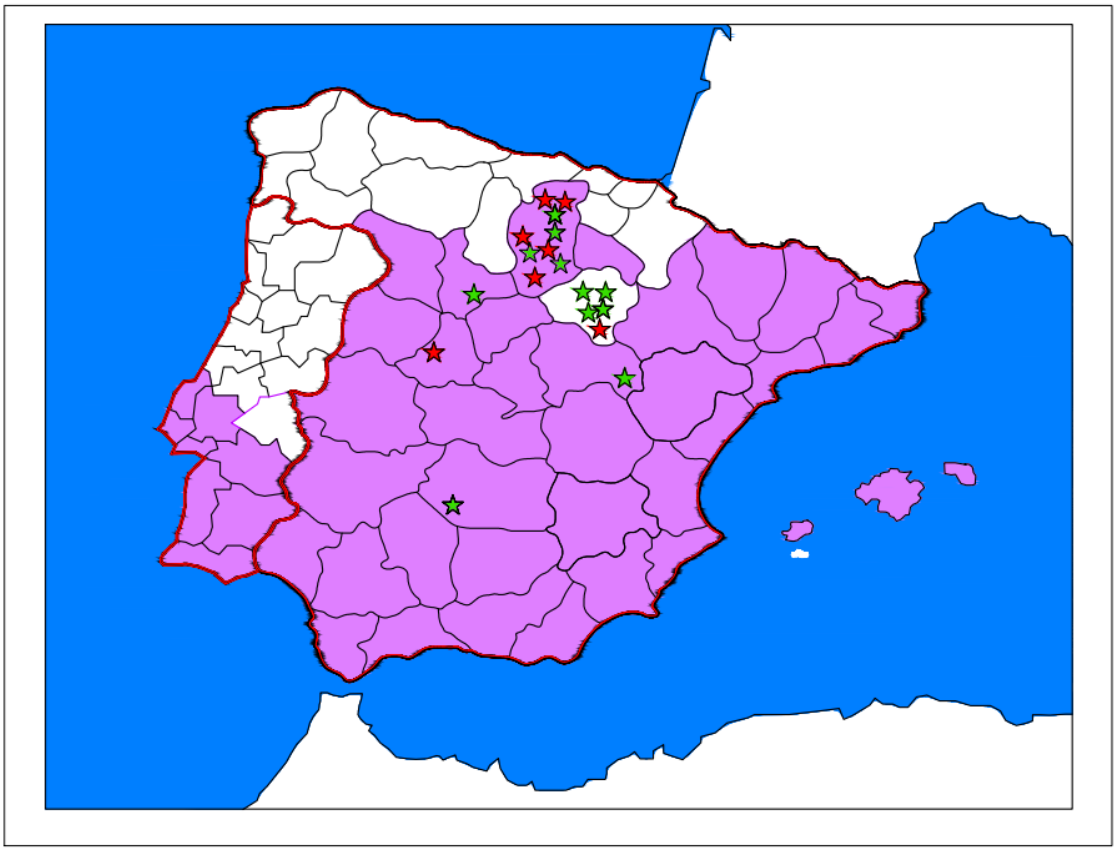

Figure 1. Map of the Iberian Peninsula. Fuchsia coloured area corresponds to the distribution of *Phlebotomus sergenti* in 2022 and 2023. Red stars indicate the detection and identification of *Leishmania* spp. by *hsp70* and *ITS1*sequences. Green stars indicate the absence of *Leishmania* spp. in the sampled cats.

Phylogenetic trees

Phylogenetic analysis of the sequences shows strong support for the clusters found with the two targets, in agreement with the percentage of identity of the obtained sequences. *ITS1* sequences from this study were grouped with *L. infantum* (samples 263, 268 and 323 from ear skin and samples 263 and 323 from spleen) or *L. tropica* (sample 275) (Figure 2).

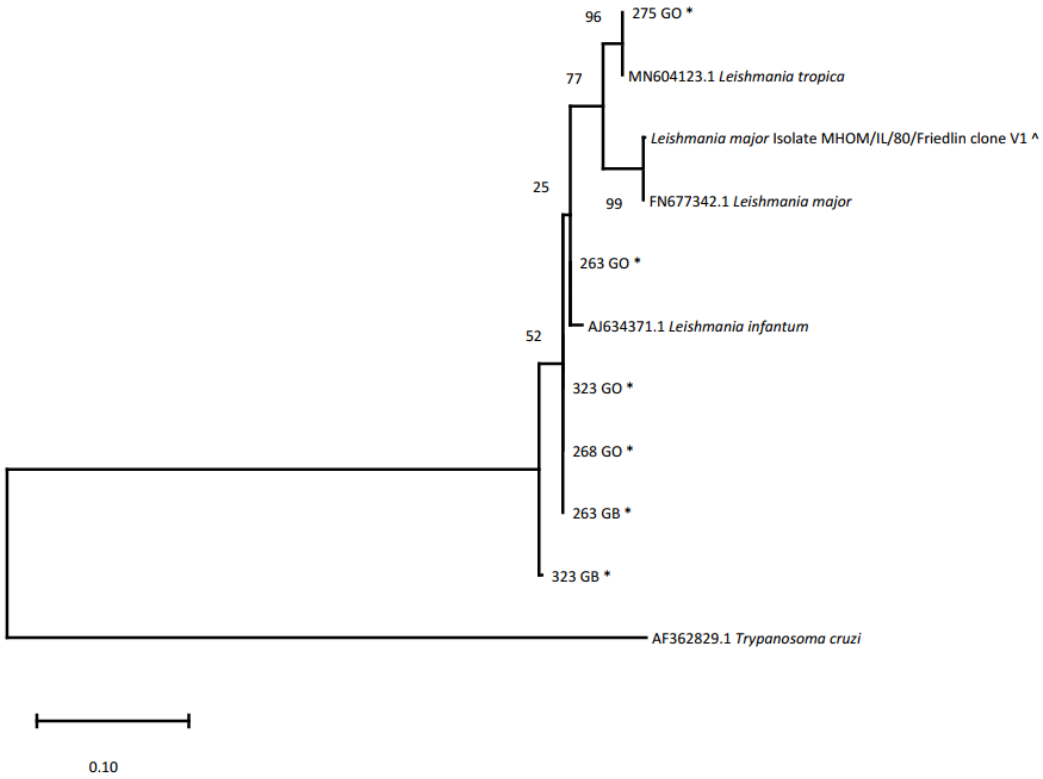

Figure 2. Phylogenetic tree of the *ITS1* region. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-841.79) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. There were a total of 292 positions in the final dataset. *****: Sequences from positive samples of this study. **^**: Sequences from isolates sequenced in the present study. GB: sample from spleen. GO: sample from ear skin.

In the phylogenetic tree generated with *hsp70* sequences obtained in the present study, two of them (samples 268 and 275 from ear skin) clustered with *L. tropica* and three of them (samples 263 and 323 from ear skin and 263 spleen) clustered with *L. infantum* sequences retrieved from GenBank (Figure 3). One of the *L. tropica* sequences was obtained from the same animal (275) that rendered *L. tropica ITS1* sequence, while the other *L. tropica hsp70* sequence was obtained from an animal (268) that rendered *L. infantum ITS1* sequence.

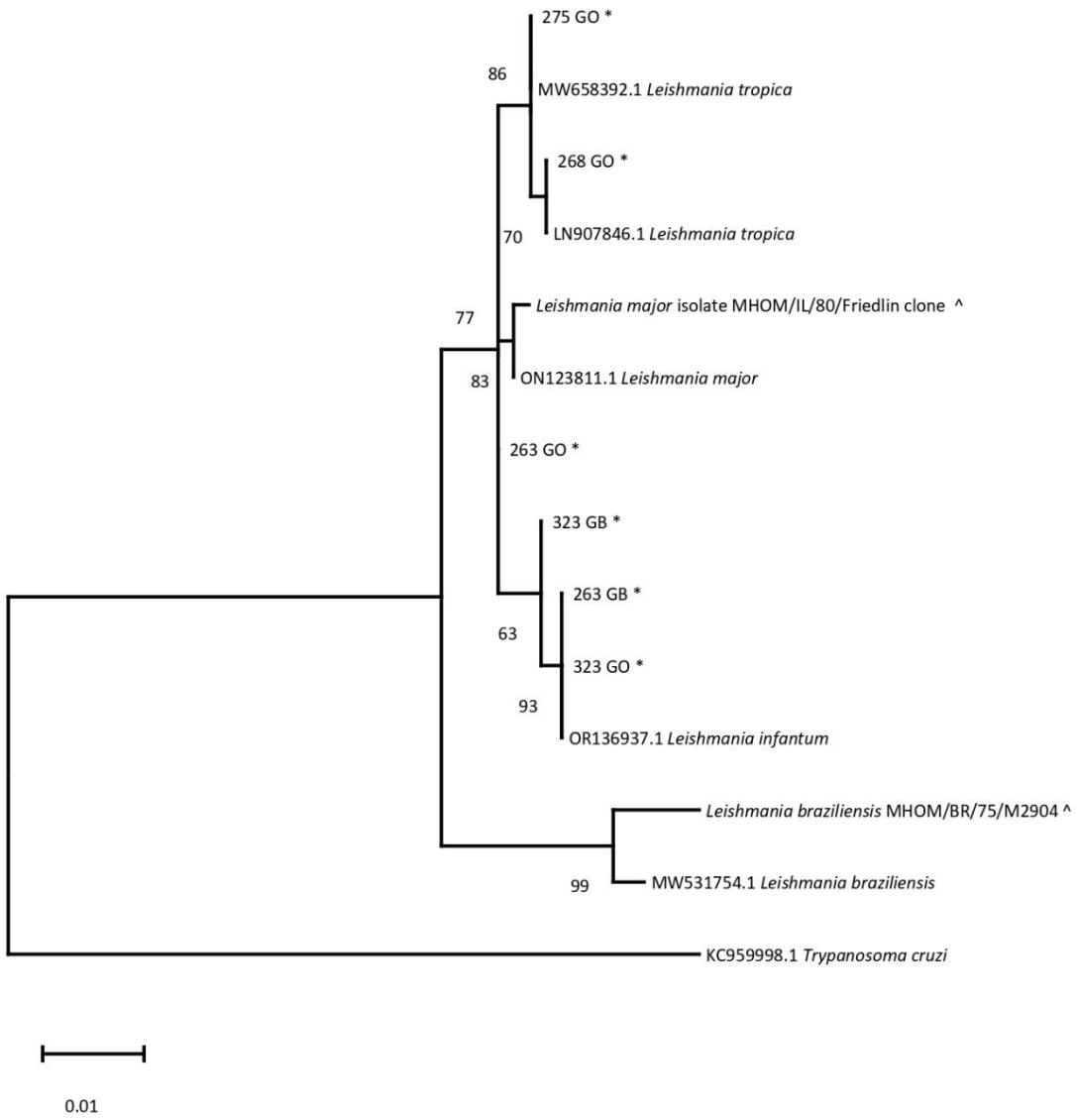

Figure 3. Phylogenetic tree of the *hsp70* region. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log-likelihood (-1328.37) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 14 nucleotide sequences. There were a total of 635 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. *****: Sequences obtained from positive samples of this study. **^**: Sequences from isolates sequenced in the present study. GB: sample from spleen. GO: sample from ear skin.

Discussion

In the present study, seven out of 18 wildcats (38.89%) were found positive to *Leishmania* infection, a value consistent with those found in previous studies [8,10] although lower than the infection prevalence reported by Oleaga et al. [9] with all three wildcats tested being positive for *Leishmania*. *L. infantum* is endemic in the Iberian Peninsula [12,13], but no infections of *L. tropica*

in autochthonous species have been reported. The risk of introduction of *L. tropica* in the Iberian Peninsula has been highlighted several times, considering the presence and distribution of the main vector, *P. sergenti*, and the identification in Spain of one genetic line of *P. sergenti* commonly found in Morocco [26,27] where CL by *L. tropica* is endemic [21,28,29]. Large areas in Europe are considered suitable for *L. tropica* [11] and hot spots for foci of *L. tropica* have been suggested [26].

The most common targets employed for detection of *Leishmania* spp. infection are *SSU-rDNA* and kDNA, with similar values of sensitivity [6]. However other targets are useful for phylogenetic studies and determination of *Leishmania* spp., such as *ITS1*, *cytB*, *g6pdh*, and *hsp70* [14,20–22,28,30,31]. In the present study, *ITS1* and *hsp70* have been employed to confirm the finding of *L. tropica* in autochthonous fauna, and sequences of *L. tropica* with *ITS1* and *hsp70* targets are reported. Sequences of *L. tropica* were detected in at least two wildcats positive to PCR with *hsp70* and/or *ITS1* targets, widely employed for phylogenetic studies. Our finding extends the reported presence of the species in Greece [32] and extends its distribution to the Iberian Peninsula for the first time.

Isolation of alive *Leishmania* was not feasible considering the source of material but it is also noteworthy than in our study ear skin samples displayed more positive results than spleen samples (n=7/18 *vs*. n=2/16). This finding is consistent with the fact that *L. tropica* infections are associated mainly with cutaneous clinical forms in humans, where parasite persistence is close to the site of natural infections transmitted by the sandfly, although the parasite can be also found less frequently associated with VL, mainly in immune suppressed people [33]. Imported cases of CL by *L. tropica* have been sporadically reported in Spain after travelling to Morocco [34] but no previous autochthonous cases have been reported in Spain or Portugal. Apparently, *L. tropica* is expanding its distribution and the main risk factor for the appearance of the disease is the presence of the vector [35]. It should be pointed out that some of the wildcats came from latitudes over 42º N (*e.g.* Pedrosa del Príncipe, N42°14'56.8") and during the last decade, *P. sergenti* has expanded to northern areas of the Iberian Peninsula, according to ECDC maps of distribution [18].

In endemic areas, such as Israel, domestic cats appear to be more susceptible to infection with *L. tropica* than dogs [36]. A study of stray cats in Turkey showed that all samples with *ITS1* amplification were infected with *L. tropica*, while only one was co-infected with *L. infantum* [37].Similarly, in the present study, while one animal showed infection by *L. tropica* only, another wildcat showed *hsp70* and *ITS1* sequences consistent with coinfection by *L. infantum* and *L. tropica*. Mixed infection with more than one *Leishmania* species have also been reported in other endemic areas, hosts and *Leishmania* species, such as in Brazil (*L. infantum* and *L. braziliensis*) in one dog [38] and 8/30 dogs [39], rodents (10% of *L. infantum* and *L. braziliensis*) [40], hedgehogs (*L. major* and *L. infantum*) in 8/12 animals and another co-infected with *L. tropica* [41], and human clinical cases [[42,43] of the New World. The possibility of hybridization between *Leishmania* species cannot be ruled out as *L. tropica* shows a higher rate of natural hybrid formation than other species [44]. However, to further investigate the existence of hybrids, isolates from infected animals are necessary [45,46], and in our study, isolates from these animals could not be obtained.

Conclusions

This is the first report of *L. tropica* infection in autochthonous wildlife in the Iberian Peninsula thus extending the presence of the species in continental Europe. The main vector of *L. tropica*

has been identified in Spain during the last decade and its distribution has increased northwards each year. There are no conclusive data on the zoonotic transmission of *L. tropica* and the actual spread of the infection to other species is not yet known and should be investigated. Our results support the sentinel value of wild species in the detection of not-previously reported infections. Moreover, the presence of *L. tropica* in Spain should be communicated to health professionals, including physicians, dermatologists, and veterinarians, considering that *L tropica* can produce both CL and VL in humans and pets. Awareness on the possibility of *L. tropica* infections and *L. infantum*/*L. tropica* coinfections is necessary to ensure appropriate diagnosis and management of clinical cases.

Ethical statement: The use of the samples for parasitological investigations was approved by regional authorisation of the Junta de Castilla y León, reference: "AB/is", File "AUES/CYL/001/2021" and of Castilla-La Mancha, reference: DGPFEN/SEN/avp_21_103_bis.

Funding statement: Research was partially funded by the UCM research group ICPVet.

Data availability: Sequences obtained in the present study have been deposited at GenBank Database under accession numbers: PP389513-PP389521 for *SSU-rDNA*, PP177368-PP177373 for *ITS1*, and PP397157-PP397162 for *hsp70*. Sequences of the *L. major* and *L. braziliensis* isolates are PP389522, PP389523, PP389533.

Ethical Committee

The employment of the samples for parasites investigation has been approved by permits of Junta de Castilla y León, reference: "AB/is", File "AUES/CYL/001/2021" and of Castilla-La Mancha, reference: DGPFEN/SEN/avp_21_103_bis.

Conflict of interest: None.

Authors' contributions:
MMB, IAC & MTG-M: conceptualization; IAC, PPM & MTG: methodology and formal analysis; MMB, PM & JC: collection of data and biological samples; IAC, MTG-M: writing the original draft; PMM, JC, IAC, MMB, MTG-M & JMA: interpretation of data; MMB & MTG-M: supervision; JMA, MMB & MTG-M: resources. All authors critically reviewed the manuscript, intellectually contribute to the content, and approved the final version.

References

1. https://www.who.int/health-topics/leishmaniasis#tab=tab_1 [Internet]. WHO: health-topics/leishmaniasis.

2. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis. 2016;10(3):1–40.

3. https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html [Internet]. 2023. WHO: neglected diseases, leishmaniasis.

4. Montoya A, Gálvez R, Checa R, Sarquis J, Plaza A, Barrera JP, et al. Latest trends in *L. infantum* infection in dogs in Spain, Part II: Current clinical management and control according to a national survey of veterinary practitioners. Parasit Vectors. 2020 Apr 21;13(1).

5. Zacarias DA, Rolão N, de Pinho FA, Sene I, Silva JC, Pereira TC, et al. Causes and consequences of higher *Leishmania infantum* burden in patients with kala-azar: a study of 625 patients. Tropical Medicine and International Health. 2017 Jun 1;22(6):679–87.

6. Azami-conesa I, Gómez-Muñoz MT, Martínez-Díaz RA. A systematic review (1990–2021) of wild animals infected with zoonotic *Leishmania*. Vol. 9, Microorganisms. MDPI AG; 2021.

7. Cardoso L, Schallig H, Persichetti MF, Pennisi MG. New Epidemiological Aspects of Animal Leishmaniosis in Europe : The Role of Vertebrate Hosts Other than Dogs. 2021;1–46.

8. Del Río L, Chitimia L, Cubas A, Victoriano I, De la Rúa P, Gerrikagoitia X, et al. Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. Prev Vet Med. 2014;113(4):430–5.

9. Oleaga A, Zanet S, Espí A, Raquel M, Macedo P De, Gortázar C, et al. *Leishmania* in wolves in northern Spain : A spreading zoonosis evidenced by wildlife sanitary surveillance. Vet Parasitol [Internet]. 2018;255(January):26–31. Available from: https://doi.org/10.1016/j.vetpar.2018.03.015

10. Risueño J, Ortuño M, Pérez-Cutillas P, Goyena E, Maia C, Cortes S, et al. Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. Vet Parasitol. 2018 Aug 15;259:61–7.

11. Cunze S, Kochmann J, Koch LK, Hasselmann KJQ, Klimpel S. Leishmaniasis in Eurasia and Africa: Geographical distribution of vector species and pathogens. R Soc Open Sci. 2019 May 1;6(5).

12. Berriatua E, Pérez-Cutillas P, González Vidal A, Briët O. European Centre for Disease Prevention and Control. The spatial relationship between the presence and absence of *Leishmania* spp. and leishmaniasis, and phlebotomine sand fly vectors in Europe and neighbouring countries. 2023; Available from: www.ecdc.europa.eu

13. Gálvez R, Montoya A, Cruz I, Fernández C, Martín O, Checa R, et al. Latest trends in *Leishmania infantum* infection in dogs in Spain, Part I: Mapped seroprevalence and sand fly distributions. Parasit Vectors. 2020 Apr 21;13(1).

14. Pereira A, Parreira R, Cristóvão JM, Castelli G, Bruno F, Vitale F, et al. Phylogenetic insights on *Leishmania* detected in cats as revealed by nucleotide sequence analysis of multiple genetic markers. Infection, Genetics and Evolution. 2020 Jan 1;77.

15. Frank K HMPFGARJ. *Leishmania tropica* and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases. Trans R Soc Trop Med Hyg. 1993;87(2):184–5.

16. Ait Kbaich M, Mhaidi I, Ezzahidi A, Dersi N, El Hamouchi A, Riyad M, et al. New epidemiological pattern of cutaneous leishmaniasis in two pre-Saharan arid provinces, southern Morocco. Acta Trop. 2017 Sep 1;173:11–6.

17. Tunalı V, Özbilgin A. Knock, knock, knocking on Europe's door: Threat of leishmaniasis in Europe with a focus on Turkey. Vol. 4, Current Research in Parasitology and Vector-Borne Diseases. Elsevier B.V.; 2023.

18. https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps [Internet]. 2023. European Centre for Disease Prevention and Control and European Food Safety Authority. Phlebotomine sandflies maps [internet]. Stockholm: ECDC; 2022, 2023. Available from:

19. Cruz I, Cañavate C, Rubio JM, Morales MA, Chicharro C, Laguna F, et al. Diagnosis A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in patients co-infected with human immuno-deficiency virus. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2002;S185–9.

20. Azami-Conesa I, Sansano-Maestre J, Martínez-Díaz RA, Gómez-Muñoz MT. Invasive species as hosts of zoonotic infections: The case of American mink (*Neovison vison*) and *Leishmania infantum*. Microorganisms. 2021 Jul 1;9(7).

21. Echchakery M, Chicharro C, Boussaa S, Nieto J, Carrillo E, Sheila O, et al. Molecular detection of *Leishmania infantum* and *Leishmania tropica* in rodent species from endemic cutaneous leishmaniasis areas in Morocco. Parasit Vectors. 2017;10(1):1–8.

22. Van Der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Esbroeck M Van, et al. Heat-shock protein 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics [Internet]. Available from: www.eurosurveillance.orghttp://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20543

23. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021 Jul 1;38(7):3022–7.

24. Tamura K& NM (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10, 512–526. Mol Biol Evol. 1993;10:512–26.

25. Ignacio de Blas. http://www.winepi.net/. Win Epi: Working in Epidemiology.

26. Barón SD, Morillas-Márquez F, Morales-Yuste M, Díaz-Sáez V, Gállego M, Molina R, et al. Predicting the risk of an endemic focus of *Leishmania tropica* becoming established in south-western Europe through the presence of its main vector, *Phlebotomus sergenti* Parrot, 1917. Parasitology. 2013;140(11):1413–21.

27. Merino-Espinosa G, Corpas-López V, Callejón-Fernández R, Porcel-Rodríguez L, Díaz-Sáez V, Gállego M, et al. Differential ecological traits of two *Phlebotomus sergenti* mitochondrial lineages in southwestern Europe and their epidemiological implications. Tropical Medicine and International Health. 2016 May 1;21(5):630–41.

28. El Kacem S, Ait Kbaich M, Daoui O, Charoute H, Mhaidi I, Ejgha R, et al. Multilocus sequence analysis provides new insight into population structure and genetic diversity of *Leishmania tropica* in Morocco. Infection, Genetics and Evolution. 2021;september:104932.

29. Saik IEI, Benlabsir C, Fellah H, Lemrani M, Riyad M. Transmission patterns of *Leishmania tropica* around the Mediterranean basin: Could Morocco be impacted by a zoonotic spillover? Vol. 16, PLoS Neglected Tropical Diseases. Public Library of Science; 2022.

30. Mohammadpour I, Hatam GR, Handjani F, Bozorg-Ghalati F, Pourkamal D, Motazedian MH. *Leishmania* cytochrome b gene sequence polymorphisms in southern Iran: Relationships with different cutaneous clinical manifestations. BMC Infect Dis. 2019 Jan 29;19(1).

31. Fernández-Arévalo A, Ballart C, Muñoz-Basagoiti J, Basarte L, Lobato G, Arnau A, et al. Autochthonous and imported tegumentary leishmaniasis in Catalonia (Spain): Aetiological evolution in the last four decades and usefulness of different typing approaches based on biochemical, molecular and proteomic markers. Transbound Emerg Dis. 2022 May 1;69(3):1404–18.

32. Frank C HMPFGARJ. *Leishmania tropica* and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases. Trans R Soc Trop Med Hyg. 1993;87(2).

33. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Vol. 27, Medical and Veterinary Entomology. 2013. p. 123–47.

34. Knöpfel N, Noguera-Morel L, Azorin D, Sanz F, Torrelo A, Hernández-Martín A. Cutaneous *Leishmania tropica* in children: report of three imported cases successfully treated with liposomal amphotericin B. Vol. 32, Journal of the European Academy of Dermatology and Venereology. Blackwell Publishing Ltd; 2018. p. e8–10.

35. Gijón-Robles P, Abattouy N, Merino-Espinosa G, El Khalfaoui N, Morillas-Márquez F, Corpas-López V, et al. Risk factors for the expansion of cutaneous leishmaniasis by *Leishmania tropica*: Possible implications for control programmes. Transbound Emerg Dis. 2018 Dec 1;65(6):1615–26.

36. Baneth G, Nachum-Biala Y, Adamsky O, Gunther I. *Leishmania tropica* and *Leishmania infantum* infection in dogs and cats in central Israel. Parasit Vectors. 2022 Dec 1;15(1).

37. Can H, Döşkaya M, Özdemir HG, Şahar EA, Karakavuk M, Pektaş B, et al. Seroprevalence of Leishmania infection and molecular detection of *Leishmania tropica* and *Leishmania infantum* in stray cats of I˙zmir, Turkey. Exp Parasitol. 2016 Aug 1;167:109–14.

38. Madeira MF, Schubach A, Schubach TMP, Pacheco RS, Oliveira FS, Pereira SA, et al. Mixed infection with *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *chagasi* in a naturally infected dog from Rio de Janeiro, Brazil. Trans R Soc Trop Med Hyg. 2006 May;100(5):442–5.

39. Alves Souza N, Souza Leite R, de Oliveira Silva S, Groenner Penna M, Figueiredo Felicori Vilela L, Melo MN, et al. Detection of mixed *Leishmania* infections in dogs from an endemic area in southeastern Brazil. Acta Trop. 2019 May 1;193:12–7.

40. Ferreira E de C, Cruz I, Cañavate C, de Melo LA, Pereira AAS, Madeira FAM, et al. Mixed infection of *Leishmania infantum* and *Leishmania braziliensis* in rodents from endemic urban area of the New World. BMC Vet Res. 2015 Mar 20;11(1).

41. Souguir-Omrani H, Chemkhi J, Fathallah-Mili A, Saadi-BenAoun Y, BelHadjAli I, Guizani I, et al. *Paraechinus aethiopicus* (Ehrenberg 1832) and *Atelerix algirus* (Lereboullet 1842) hedgehogs: Possible reservoirs of endemic leishmaniases in Tunisia. Infection, Genetics and Evolution. 2018 Sep 1;63:219–30.

42. Shirian S, Oryan A, Hatam GR, Daneshbod Y. Mixed mucosal leishmaniasis infection caused by *Leishmania tropica* and *Leishmania major*. J Clin Microbiol. 2012 Nov;50(11):3805–8.

43. Badirzadeh A, Mohebali M, Sabzevari S, Ghafoori M, Arzamani K, Seyyedin M, et al. Case Report: First coinfection report of mixed *Leishmania infantum*/*Leishmania major* and human immunodeficiency virus-acquired immune deficiency syndrome: Report of a case of disseminated cutaneous Leishmaniasis in Iran. American Journal of Tropical Medicine and Hygiene. 2018;98(1):122–5.

44. Louradour I, Ferreira TR, Ghosh K, Shaik J, Sacks D. In Vitro Generation of *Leishmania* Hybrids. Cell Rep. 2020 Apr 14;31(2).

45. Ozbilgin A, Culha G, Zeyrek FY, Töz S, Gündüz C, Kurt Ö, et al. Cutaneous and visceral tropisms of *Leishmania tropica*/*Leishmania infantum* hybrids in a murine model: First report of hybrid *Leishmania* strains isolated in Turkey. International Journal of Infectious Diseases. 2012 Jun;16:e165.

46. Bruno F, Castelli G. Genomic and epidemiological evidence for the emergence of a putative *L. donovani*/*L. infantum* hybrid with unusual epidemiology in Northern Italy. Available from: https://doi.org/10.1101/2023.08.09.552585