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Roe deer (*Capreolus capreolus*) are a novel potential reservoir for human visceral leishmaniasis in the Emilia-Romagna region of northeastern Italy ☆

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ABSTRACT

Leishmaniasis is a complex human disease caused by intracellular parasites of the genus *Leishmania,* predominantly transmitted by the bite of sand flies. In Italy, leishmaniasis is caused exclusively by *Leishmania infantum*, responsible for the human and canine visceral leishmaniases (HVL and CVL, respectively). Within the Emilia-Romagna region, two different foci are active in the municipalities of Pianoro and Valsamoggia (both in the province of Bologna). Recent molecular studies indicated that *L. infantum* strains circulating in dogs and humans are different, suggesting that there is an animal reservoir other than dogs for human visceral leishmaniasis in the Emilia-Romagna region. In this work, we analyzed specimens from wild animals collected during hunts or surveillance of regional parks near active foci of human visceral leishmaniasis for *L. infantum* infection in the province of Bologna. Out of 70 individuals analyzed, 17 (24%) were positive for *L. infantum*. The infection prevalence in hedgehogs (*Erinaceus europaeus*), roe deer (*Capreolus capreolus*), badgers (*Meles meles*), and bank voles (*Myodes glareolus*) was 80, 33, 25, and 11%, respectively. To distinguish the two strains of *L. infantum* we have developed a nested PCR protocol optimized for animal tissues. Our results demonstrated that most (over 90%) of *L. infantum* infections in roe deer were due to the strain circulating in humans in the Emilia-Romagna region.

1. Introduction

Leishmaniasis is a complex of tropical and subtropical vector-borne diseases with up to 1 million new cases recorded annually (WHO, 2022. Leishmaniasis, https://www.who.int/en/news-room/-fact-sheets/detail/leishmaniasis. (accessed August 16, 2022)). It is caused by sandfly-transmitted parasitic flagellates of the genus *Leishmania* (Euglenozoa: Kinetoplastea: Trypanosomatidae), whose development in vertebrates occurs intracellularly (Kostygov et al., 2021). The disease has three main clinical forms: cutaneous (skin sores), mucocutaneous (destruction of skin and mucosa), and visceral (systemic inflammation focusing on liver, spleen, and bone marrow) (Bruschi and Gradoni, 2018). The latter form is the most dangerous as mortality in untreated patients can exceed 95% (WHO, 2022. Leishmaniasis, https://www.who.int/en/news-room/-fact-sheets/detail/leishmaniasis. (accessed August 16, 2022)). Leishmaniasis in Italy is caused only by *Leishmania infantum* (Lukeš et al., 2007), which is responsible for human and canine visceral leishmaniases (HVL and CVL, respectively). In the Emilia-Romagna region (ER) of northeastern Italy, HVL outbreaks have been described since the 1970s, mostly in the foothill areas (Pampiglione, 1975). From the 1990s, the outbreaks became recurrent and *L. infantum* was documented even in the non-endemic territories of northern Italy (Maroli et al., 2008; Abdalmaula et al., 2013; Cesinaro et al., 2017; Ferroglio et al., 2018). Currently in the Bologna province of ER, two different foci are active in the municipalities of Pianoro and Valsamoggia (Varani et al., 2013; Ortalli et al., 2020).

The HVL caused by *L. infantum* is considered zoonotic, i.e. mammals other than humans are always involved in circulation of the parasite (Bruschi and Gradoni, 2018). For example, in Italy, dogs were proposed for that role, however, a wide range of wildlife species have also been implicated as potential reservoirs in Europe: carnivores (canids, felids, mustelids, badgers, etc.), lagomorphs

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank under accession numbers **OP186448-OP186451**.

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(hares and rabbits), insectivores (hedgehogs and shrews), rodents (various mice and rats, squirrels, etc.), and bats (Bruschi and Gradoni, 2018; Cardoso et al., 2021). Wild ruminants might also serve as reservoirs, but they have been sampled rather scarcely. To the best of our knowledge, there were no reports on *L. infantum* infections in these animals either in Europe, or on other continents thus far.

Recent studies demonstrated that the *L. infantum* population in ER is heterogeneous and can be split into two different strains which are exclusively present (besides sandflies) in either human patients or dogs (Rugna et al., 2017, 2018). Notably, in other regions of Italy, the parasites of humans are genetically similar to those that infect dogs in the ER region. This implies that parasites causing HVL in ER have distinct animal reservoirs, as initially suggested in 1974 (Pampiglione et al., 1974). In line with this, analysis of the vectors' blood meal preferences in this area showed a strong bias toward wild mammals (Calzolari et al., 2022).

In the present study, 70 individuals of nine species of wild mammals collected in municipalities of Bologna located near the active foci of HVL were screened for the presence of *Leishmania* spp. This was done using real-time PCR for kinetoplast DNA (kpDNA) as well as a newly developed nested PCR protocol allowing precise strain differentiation. In addition to the known reservoirs such as mice, rats and hedgehogs, we detected *L. infantum* in roe deer, thereby presenting the first known report of such an infection in ruminants.

2. Materials and methods

2.1. Collected material

From June 2019 to December 2020, organs and entire carcasses of 70 wild mammals belonging to nine species were sampled near active HVL foci in the Pianoro and Valsamoggia municipalities of Bologna (Table 1; Fig. 1). In particular, ear lobes and spleen from roe deer, as well as carcasses of hares, were provided by professional hunters, while carcasses of other mammals revealed during park surveillance were collected by volunteers and park rangers. When entire carcasses were available, four samples were taken from each of them during necropsies: the ear lobe skin, spleen, liver, and prescapular lymph nodes. The necropsy details have been described elsewhere (Magri et al., 2022). DNA from these samples was isolated with PureLink® Genomic DNA Mini Kit (Invitrogen/ Thermo Fisher Scientific, Carlsbad, USA) according to the manufacturer's instructions.

2.2. Real-time PCR

The presence of *Leishmania* spp. was assessed with a highly sensitive real-time PCR targeting a 71-bp region of minicircle kpDNA using primer pair Leish71Up (5′-ccaaacttttctggtcctycgggtag-3′) and Leish71Do (5′-tgaacgggatttctgcacccatttttc-3′) (Tsakmakidis et al., 2017). The details of amplification and parasite quantification were described previously Magri et al. (2022).

Table 1
Summary of the samples collected in Italy.

Species	Locality	Individuals positive/total
Roe deer (*Capreolus capreolus*)	Pianoro	11/33
Hare (*Lepus europaeus*)		0/13
Hedgehog (*Erinaceus europaeus*)		3/3
	Valsamoggia	1/2
Badger (*Meles meles*)		1/4
Bank vole (*Myodes glareolus*)		1/9
Beech marten (*Martes foina*)		0/1
European polecat (*Mustela putorius*)		0/1
Common shrew (*Sorex araneus*)		0/3
Fox (*Vulpes vulpes*)		0/1

2.3. Nested PCR

Discrimination of *L. infantum* strains based on the one-step amplification of the gene encoding cysteine peptidase B (*cpb*), featuring a 39-nucleotide (nt) indel, was previously proposed for laboratory cultures (Zackay et al., 2013; Rugna et al., 2017). However, application of this strategy to tissue samples resulted in multiple PCR by-products originating from host DNA and prevented diagnosis of *Leishmania* infection. To overcome this problem, we developed a nested PCR protocol by adding a second pair of primers annealing within the amplicon produced at the first amplification stage. It is important to note that the *L. infantum* genome contains multiple copies of the *cpb* gene, of which only one varies as described above. This copy has a distinct sequence at the 3′ end allowing its specific amplification. The *cpb* sequences were retrieved from GenBank (accession numbers: AJ628943, AY896776, AY896777, AY896778, AY896780, AY896782, AY896791, EU637907, GQ302670, GQ302671, GQ302674, GQ856074, JN400122-JN400131, XM_001463394). For the first round of PCR, previously reported primers cpbEFF (5′-gttatggctgc gtggcttg-3′) and cpbEFR (5′-cgtgcactcggccgtctt-3′) were used (Zackay et al., 2013). For the second round, a new primer pair was designed using Geneious Prime (Dotmatics, Boston, USA) software: cpbt1: 5′-tgtccagcatgcctcacaaga-3′ and cpbt2: 5′-ccagctcctt catgtcttacca-3′ (Fig. 2).

Leishmania infantum strain identity was determined for 17 samples which tested positive here by real-time PCR and for 15 previously reported positive samples from black rats (*Rattus rattus*), brown rats (*Rattus norvegicus*), and mice (*Mus musculus*) collected in the ER region (Magri et al., 2022). Reactions were carried out in a total volume of 25 µl with 12.5 µl of PCRBIO Taq Mix Red (PCR Biosystems ltd, London, UK), 0.3 µM of each primer and 2 µl of DNA in the first round and 1.5 µl of template in the second round. For both rounds, amplification was performed as follows: initial denaturation 94 °C for 4 min, followed by 30 cycles 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min and 72 °C for 5 min final elongation. As a positive control, the reference strain *L. infantum* MHOM/TN/80/IPT1 was used. The amplified fragments were separated on a 1.5% agarose gel stained with Midori Green Advance (Nippon Genetics Europe, Düren, Germany). The fragment lengths were 281 bp (long – L variant, no deletion) or 242 bp (short – S variant, deletion) (Fig. 3). The identity of the PCR products was confirmed by sequencing four samples (two long and two short). The Sample Size Calculator (https://www.calculator.net/sample-size-calculator.html) was used to calculate 95% confidence intervals (CIs) for the observed prevalence values.

3. Results

3.1. Real-time PCR

In the real-time PCR screening, 17 out of 70 analyzed individuals (24% overall prevalence) tested positive (Table 2). The presence of leishmaniae was detected in earlobe skin of 11 roe deer out of 33 analyzed (prevalence 33%; 95% CI: 17.3, 49.4). In one of these samples, the parasites were also detected in the spleen. Four out of five hedgehogs (prevalence 80%; 95% CI: 44.9, 100), showed signal either in the spleen or in the ear lobe skin (three and one individual, respectively). Leishmaniae were also detected in the liver and spleen of one bank vole (prevalence 11%; 95% CI: 0, 31.6 as well as

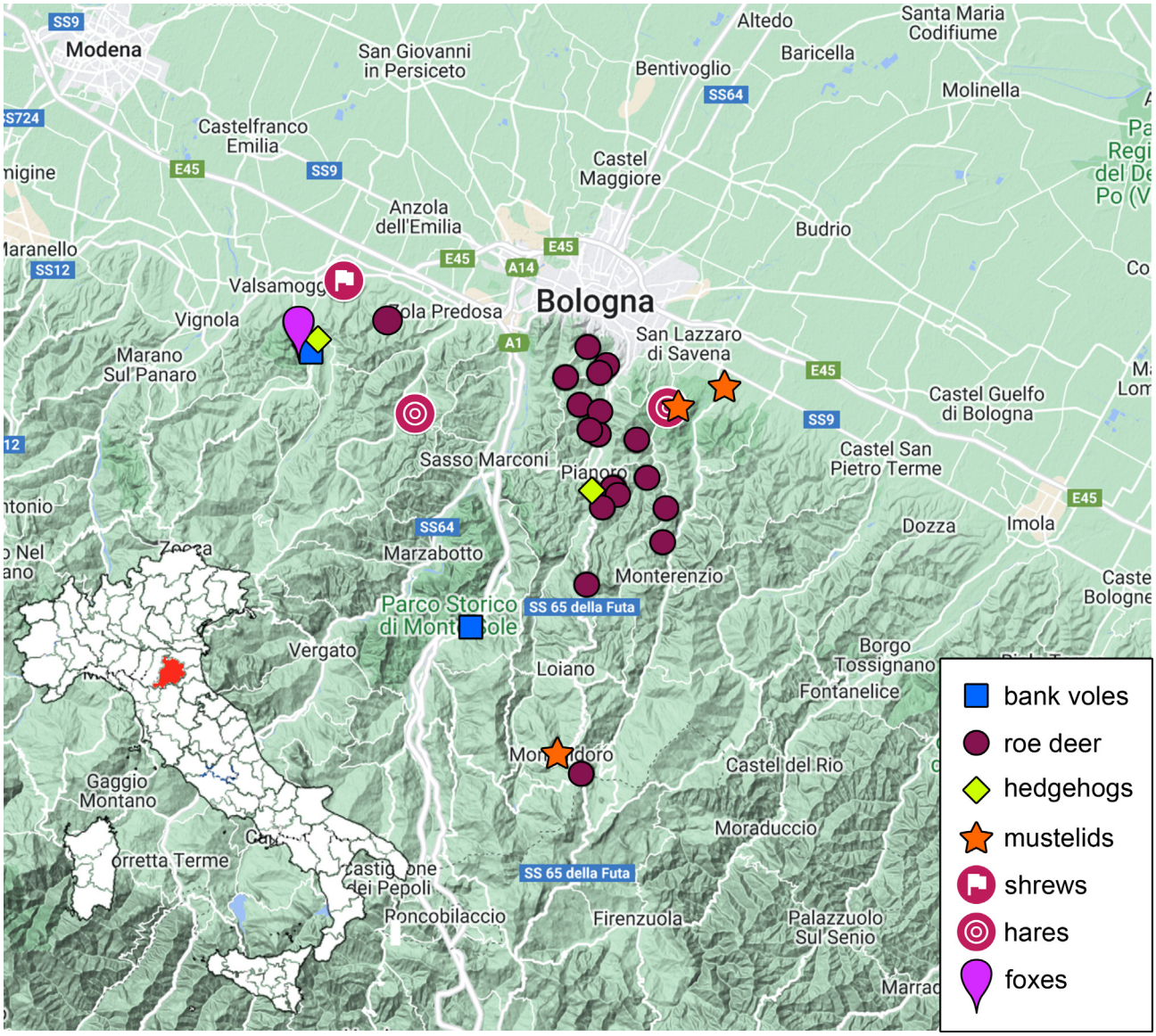

Fig. 1. Map of the region in Italy where the samples were collected.

in the liver of a single badger (prevalence 25%; 95% CI: 0, 67.4) (Table 2).

3.2. Nested PCR

The 17 samples which tested positive in the present study, and 15 isolates from mice and two species of rats from which *Leishmania* has been detected in a previous study (Magri et al., 2022), were subjected to strain identification by the newly developed nested PCR. For seven out of the combined 32 samples, the *Leishmania* strain identity could not be determined due to the lack of PCR products. This apparently can be explained by the low content of parasite DNA and/or its degradation due to tissue decomposition. The real-time PCR used here is more resistant to the latter issue, since it targets a much shorter fragment (71 bp versus 365–404 bp amplified at the first step of the nested PCR). Moreover, the kpDNA fragment originates from a multicopy template (minicircles) compared with the single-copy nuclear gene target of the nested PCR. For the remaining 25 samples, the *L. infantum* strain identity was determined as L (long) or S (short). The L and S genotypes in our work correspond to the *cbp*F (documented in humans and sandflies in the ER region) and *cbp*E (documented in dogs in the ER region and humans elsewhere) genotypes, respectively (Rugna et al., 2017, 2018). In total, we documented 16 L and nine S genotypes. The host species distribution for the analyzed genotypes was as follows: roe deer – 10 L (91%), one S (9%); hedgehogs – three L (75%) and one S (25%); mice – two L (40%), one S (20%), and two samples were unidentified due to PCR failure; black rats – one L (20%), three S (60%), and one unidentified sample; brown rats – no L, three S (60%) and two unidentified samples.

4. Discussion

In Europe, several studies aimed to assess the presence of *L. infantum* in wild and peri-domestic animals. Even though the role of these animals as reservoirs has been demonstrated in certain outbreaks (Molina et al., 2012; Helhazar et al., 2013; Tsakmakidis et al., 2017), it is still uncertain if they can also act as accidental hosts or amplifiers (Tomassone et al., 2018).

In the current study, samples from 70 individuals of nine wildlife species, collected in the proximity of HVL foci of the ER region,

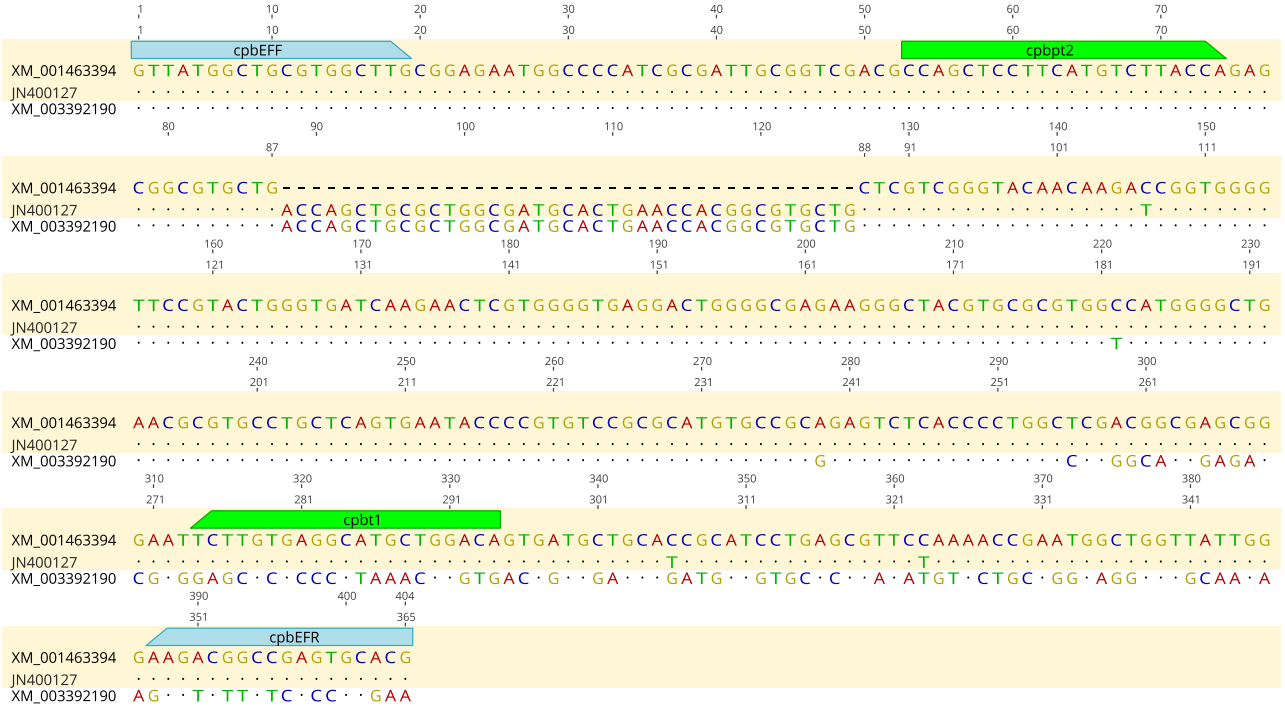

Fig. 2. Alignment of partial cysteine peptidase B gene sequences of *Leishmania infantum* demonstrating the annealing sites of the used primers and the position of the indel. The target gene variant (highlighted) in the strains JPCM5 and Drep 13 is shown together with a non-target variant of the strain JPCM5 (GenBank accession numbers XM_001463394, JN400127, and XM_003392190, respectively). Nucleotides identical to those in the first sequence are replaced with dots. Dashes show the characteristic deletion allowing strain discrimination. The PCR products obtained from XM_001463394 and JN400127 are classified as *cbp*E and *cbp*F genotypes, respectively, when amplified with the external primers (in blue), or as S and L genotypes when amplified with the internal primers (in green).

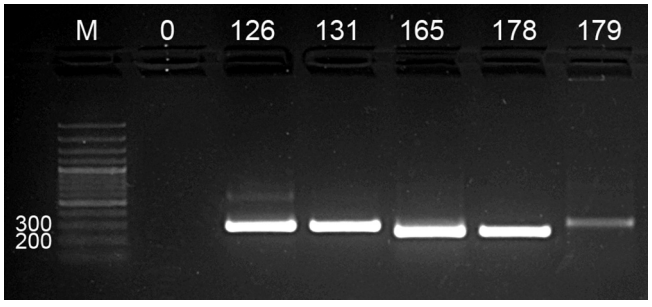

Fig. 3. Nested PCR detection of the L (specimens 126, 131, and 179) and S (specimens 165 and 178) genotypes of *Leishmania infantum*. The 100-bp ladder (Thermo Fisher Scientific, Waltham, USA) is on the left. Lane "0" is a negative amplification control.

were screened for *Leishmania* spp. and 17 of them tested positive (prevalence 24%). Of particular interest is the fact that approximately one-third of the examined roe deer were positive. Moreover, the majority of them (10/11) harbored the strain associated with HVL in ER. This suggests that roe deer may represent a natural reservoir of *L. infantum* in general and HVL in the ER region in particular.

The parasite load, especially in the skin, can reflect infectiousness in the natural life cycle (Courtenay et al., 2014). It was previously estimated that in order to serve as a reservoir, the host species should demonstrate an infection prevalence over 20% with the parasite detectable in blood or skin in sufficient amounts to be ingested by a sand fly (Roque and Jansen, 2014; Alemayehu and Alemayehu, 2017). A reservoir species should be sufficiently abundant and long-lived, thereby providing sufficient frequency of contacts with the vector (WHO, 2022). In Europe (as in the rest of the Old World), leishmaniae are mainly transmitted by *Phlebotomus* spp. (Torres-Guerrero et al., 2017). Even though these sandflies have been considered generally opportunistic (De Colmenares et al., 1995), the analysis of blood meal of *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* in the ER region revealed a high presence of roe deer blood (Calzolari et al., 2022). Taken together, these findings suggest the role of roe deer in the epidemiology of *L. infantum* in northern Italy. Whether other wild animals such as badgers, hedgehogs or bank voles may play a similar role remains to be investigated further, because the number of specimens analyzed thus far is not sufficient to make any solid conclusions.

In order to differentiate *L. infantum* strains circulating in human and animals, we took advantage of the *cpb* locus possessing a 39-bp deletion in some isolates (Hide and Bañuls, 2006; Chaouch et al., 2013). It has been previously reported that in the ER region the autochthonous human isolates were endowed with a longer sequence, while those circulating in dogs possessed the above-mentioned deletion, implying that causative agents of HVL and CVL in this region are different (Rugna et al., 2017, 2018). Here, we report that most of the wild animals collected in the proximity of active HVL foci in the ER region (10 roe deer, three hedgehogs) tested positive for the strain associated with humans. This strain has been previously documented in one mouse and one black rat from the Romagna area where, according to the leishmaniasis regional control authority, cases of the autochthonous HVL were reported last year (Santi et al., 2022). The strain circulating in dogs in the ER region was documented in one hedgehog and one roe deer in the same area, and in rodents from the Ravenna province, an area with cases of CVL in kennels.

In conclusion, we revealed two strains of *L. infantum* circulating in the wild and synanthropic fauna of the ER region of Italy. The strain causing HVL in ER was documented in roe deer collected in the proximity of active foci of this disease and represented over

Table 2
Summary of the real-time and nested PCR results (positive samples only).

ID	Species	Real-Time PCR results[b]				*cpb* ID[c]
		Earlobe Skin	Spleen	Liver	lymph Node	
24	Roe deer	32.62 (92)	–	N.A.	N.A.	L
25	Roe deer	29.48 (771)	29.97 (553)	N.A.	N.A.	S
28	Roe deer	36.07 (9)	–	N.A.	N.A.	L
29	Roe deer	32.02 (138)	–	N.A.	N.A.	L
30	Roe deer	38.27 (11)	–	N.A.	N.A.	L
34	Roe deer	28.72 (1290)	–	N.A.	N.A.	L
35	Roe deer	38.07 (2)	–	N.A.	N.A.	L
36	Roe deer	27.29 (3399)	–	N.A.	N.A.	L
123	Roe deer	33.24 (51)	–	N.A.	N.A.	L
126	Roe deer	30.6 (361)	–	N.A.	N.A.	L
131	Roe deer	33.5 (61)	–	N.A.	N.A.	L
165	Hedgehog	36.78 (6)	–	–	–	S
181	Hedgehog	–	35.66 (12)	–	–	L
182	Hedgehog	–	36.65 (6)	–	–	L
183	Hedgehog	–	34.04 (33)	–	–	L
192	Bank vole	–	35.26 (15)	33.69 (45)	–	–
193	Badger	–	–	34.144 (33)	–	–
57[a]	Mouse	32.7 (87)	29.68 (676)	–	–	L
59[a]	Mouse	–	30.77 (316)	31.97 (143)	–	–
67[a]	Mouse	–	–	–	33.61 (47)	–
98[a]	Mouse	–	36.71 (5.8)	–	37.07 (4.5)	S
111[a]	Mouse	–	–	35.9 (10)	–	L
4[a]	Brown rat	34.25 (30.9)	–	–	–	–
86[a]	Brown rat	–	–	36.47 (6.8)	–	S
141[a]	Brown rat	37.75 (2.9)	–	–	–	S
175[a]	Brown rat	–	–	36.27 (7.8)	–	–
178[a]	Brown rat	–	–	36.67 (5.8)	–	S
37[a]	Black rat	–	36.47 (6.8)	–	–	S
60[a]	Black rat	36.86 (6.2)	–	–	–	S
95[a]	Black rat	–	37.44 (6.2)	–	–	S
179[a]	Black rat	–	–	–	36.63 (6.3)	L
206[a]	Black rat	37.89 (2.6)	–	–	–	–

[a] Samples analyzed by real-time PCR in the previous study (Magri et al., 2022).
[b] Real-time PCR results are reported as Ct values averaged for triplicates with the estimated quantity of parasites/ml in parentheses; dash, amplification failure; N.A., specimen not available.
[c] Strains are classified by *cpb* (gene encoding cysteine peptidase B) PCR product length: S, short; L, long.

90% of *L. infantum* infections in these animals. This, together with other facts (predominant parasite localization in the skin facilitating transmission and the preference for roe deer by sand flies in the analyzed area) implies that roe deer can serve as a reservoir of HVL. The role of other potential wildlife reservoir species remains to be investigated further.

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References

Abdalmaula, G.H., Barbadoro, P., Marigliano, A., Illuminati, D., Di Stanislao, F., D'Errico, M.M., Prospero, E., 2013. Human visceral leishmaniasis: a picture from Italy. J. Infect. Public Health 6, 465–472.
Alemayehu, B., Alemayehu, M., 2017. Leishmaniasis: a review on parasite, vector and reservoir host. Health Sci. J. 11, 519.
Bruschi, F., Gradoni, L., 2018. The Leishmaniases: Old Neglected Tropical Diseases. Springer, Cham, Switzerland.
Calzolari, M., Romeo, G., Bergamini, F., Dottori, M., Rugna, G., Carra, E., 2022. Host preference and *Leishmania infantum* natural infection of the sand fly *Phlebotomus perfiliewi* in northern Italy. Acta Trop. 226, 106246.
Cardoso, L., Schallig, H., Persichetti, M.F., Pennisi, M.G., 2021. New epidemiological aspects of animal leishmaniosis in Europe: the role of vertebrate hosts other than dogs. Pathogens 10, 307.
Cesinaro, A.M., Nosseir, S., Mataca, E., Mengoli, M.C., Cavatorta, C., Gennari, W., 2017. An outbreak of cutaneous leishmaniasis in Modena province (Northern Italy): report of 35 cases. Pathologica 109, 363–367.
Chaouch, M., Fathallah-Mili, A., Driss, M., Lahmadi, R., Ayari, C., Guizani, I., Ben Said, M., Benabderrazak, S., 2013. Identification of Tunisian *Leishmania* spp. by PCR amplification of cysteine proteinase B (*cpb*) genes and phylogenetic analysis. Acta Trop. 125, 357–365.
Courtenay, O., Carson, C., Calvo-Bado, L., Garcez, L.M., Quinnell, R.J., 2014. Heterogeneities in *Leishmania infantum* infection: using skin parasite burdens to identify highly infectious dogs. PLoS Negl. Trop. Dis. 8, e2583.
De Colmenares, M., Portús, M., Botet, J., Dobaño, C., Gállego, M., Wolff, M., Seguí, G., 1995. Identification of blood meals of *Phlebotomus perniciosus* (Diptera: Psychodidae) in Spain by a competitive enzyme-linked immunosorbent assay biotin/avidin method. J. Med. Entomol. 32, 229–233.
Ferroglio, E., Battisti, E., Zanet, S., Bolla, C., Concialdi, E., Trisciuoglio, A., Khalili, S., Biglino, A., 2018. Epidemiological evaluation of *Leishmania infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy. Zoonoses Public Health 65, 675–682.
Helhazar, M., Leitao, J., Duarte, A., Tavares, L., da Fonseca, I.P., 2013. Natural infection of synathropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania infantum* in Sesimbra and Sintra Helhazar – Portugal. Parasit. Vectors 6, 88.
Hide, M., Bañuls, A.L., 2006. Species-specific PCR assay for *L. infantum*/*L. donovani* discrimination. Acta Trop. 100, 241–245.
Kostygov, A.Y., Karnkowska, A., Votýpka, J., Tashyreva, D., Maciszewski, K., Yurchenko, V., Lukeš, J., 2021. Euglenozoa: taxonomy, diversity and ecology, symbioses and viruses. Open Biol. 11, 200407.
Lukeš, J., Mauricio, I.L., Schönian, G., Dujardin, J.C., Soteriadou, K., Dedet, J.P., Kuhls, K., Tintaya, K.W., Jirků, M., Chocholová, E., Haralambous, C., Pratlong, F., Oborník, M., Horák, A., Ayala, F.J., Miles, M.A., 2007. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proc. Natl. Acad. Sci. U. S. A. 104, 9375–9380.

Magri, A., Galuppi, R., Fioravanti, M., Caffara, M., 2022. Survey on the presence of *Leishmania* sp. in peridomestic rodents from the Emilia-Romagna Region (North-Eastern Italy). Vet. Res. Commun. https://doi.org/10.1007/s11259-11022-09925-11254.

Maroli, M., Rossi, L., Baldelli, R., Capelli, G., Ferroglio, E., Genchi, C., Gramiccia, M., Mortarino, M., Pietrobelli, M., Gradoni, L., 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop. Med. Int. Health 13, 256–264.

Molina, R., Jiménez, M.I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet. Parasitol. 190, 268–271.

Ortalli, M., De Pascali, A.M., Longo, S., Pascarelli, N., Porcellini, A., Ruggeri, D., Randi, V., Procopio, A., Re, M.C., Varani, S., 2020. Asymptomatic *Leishmania infantum* infection in blood donors living in an endemic area, northeastern Italy. J. Infect. 80, 116–120.

Pampiglione, S., 1975. Visceral leishmaniasis in Emilia-Romagna. Ann. Sanita. Pubblica 35, 1021–1028. in Italian.

Pampiglione, S., La Placa, M., Schlick, G., 1974. Studies on mediterranean leishmaniasis. I. An outbreak of visceral leishmaniasis in Northern Italy. Trans. R. Soc. Trop. Med. Hyg. 68, 349–359.

Roque, A.L., Jansen, A.M., 2014. Wild and synanthropic reservoirs of *Leishmania* species in the Americas. Int. J. Parasitol. Parasites Wildl. 3, 251–262.

Rugna, G., Carra, E., Corpus, F., Calzolari, M., Salvatore, D., Bellini, R., Di Francesco, A., Franceschini, E., Bruno, A., Poglayen, G., Varani, S., Vitale, F., Merialdi, G., 2017. Distinct *Leishmania infantum* strains circulate in humans and dogs in the Emilia-Romagna region, Northeastern Italy. Vector Borne Zoonotic Dis. 17, 409–415.

Rugna, G., Carra, E., Bergamini, F., Calzolari, M., Salvatore, D., Corpus, F., Gennari, W., Baldelli, R., Fabbi, M., Natalini, S., Vitale, F., Varani, S., Merialdi, G., 2018. Multilocus microsatellite typing (MLMT) reveals host-related population structure in *Leishmania infantum* from northeastern Italy. PLoS Negl. Trop. Dis. 12, e0006595.

Santi, A., Rossi, A., Rocca, R., Galletti, G., Casadei, G., Tamba, M., 2022. [Epidemiological surveillance. Emilia-Romagna region. Regional leishmaniasis control plan - results for the year 2021], Sorveglianza Epidemiologica Emilia Romagna (SEER). Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Bologna, Italy. (in Italian).

Tomassone, L., Berriatua, E., De Sousa, R., Duscher, G.G., Mihalca, A.D., Silaghi, C., Sprong, H., Zintl, A., 2018. Neglected vector-borne zoonoses in Europe: into the wild. Vet. Parasitol. 251, 17–26.

Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J., Arenas, R., 2017. Leishmaniasis: a review. F1000Res 6, 750.

Tsakmakidis, I., Angelopoulou, K., Dovas, C.I., Dokianakis, E., Tamvakis, A., Symeonidou, I., Antoniou, M., Diakou, A., 2017. *Leishmania* infection in rodents in Greece. Trop. Med. Int. Health 22, 1523–1532.

Varani, S., Cagarelli, R., Melchionda, F., Attard, L., Salvadori, C., Finarelli, A.C., Gentilomi, G.A., Tigani, R., Rangoni, R., Todeschini, R., Scalone, A., Di Muccio, T., Gramiccia, M., Gradoni, L., Viale, P., Landini, M.P., 2013. Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013. Euro. Surveill. 18, 20530.

Zackay, A., Nasereddin, A., Takele, Y., Tadesse, D., Hailu, W., Hurissa, Z., Yifru, S., Weldegebreal, T., Diro, E., Kassahun, A., Hailu, A., Jaffe, C.L., 2013. Polymorphism in the HASPB repeat region of East African *Leishmania donovani* strains. PLoS Negl. Trop. Dis. 7, e2031.