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RESEARCH ARTICLE

Epidemiological landscape in a Mediterranean hotspot of human leishmaniosis in Spain under a One Health approach

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ABSTRACT

Integrated One Health studies are essential to assess the potential risks associated with leishmaniosis hotspots in Europe. Thus, the aim was to holistically evaluate *Leishmania infantum* epidemiology in a rural hotspot in Spain with a high incidence of human leishmaniosis. Samples from 145 humans (blood), 41 dogs (blood and hairs), and 41 wild lagomorphs (blood, skin, and spleen) were collected during 2022–2023. Sandflies were captured with CDC-traps, and blood-feeding was evaluated. *L. infantum* exposure was assessed using indirect immunofluorescence and/or quantitative PCR. Positivity was detected in 6.2% of humans, 73.2% of dogs, and 100% of lagomorphs. A total of 1,347 sandflies were captured, predominantly *Phlebotomus perniciosus*. Blood meal analysis identified several synanthropic animals, as well as humans, as blood-sources. *L. infantum* DNA was detected in 65.7% of pooled and 25.8% of individual sandfly specimens. A spatial cluster of *L. infantum* positivity was identified near a hunting area harboring lagomorphs. Phylogeny revealed high homology between *L. infantum* isolates from lagomorphs and sandflies. Our results reinforce the role of wild lagomorphs as pivotal *L. infantum* reservoirs, favoring the occurrence of human leishmaniosis at the wildlife-human-domestic interface. This study underscores the need to integrate One Health approaches in endemic areas of leishmaniosis to establish effective prevention and control measures.

KEYWORDS

Dog; hotspot; human; *Leishmania*; sandfly; wild lagomorphs

Introduction

Leishmaniosis is a neglected vector-borne disease caused by different species of the genus *Leishmania* that is transmitted through the bite of infected sandflies [1]. This zoonosis is endemic in 99 countries where more than one billion people are at risk of infection; it causes approximately one million new cases and 30,000 deaths worldwide each year [2]. In humans, three clinical forms of leishmaniosis are reported: visceral leishmaniosis (VL), cutaneous leishmaniosis (CL), and mucocutaneous leishmaniosis (MCL) [2]. Although human leishmaniosis is a notifiable disease in most endemic European countries, it is an underdiagnosed and underreported disease causing between 1,000–-1,900 and 10,000–17,000 cases of VL and CL, respectively, in Europe each year [3].

In Europe, *Leishmania infantum* is the main circulating *Leishmania* species responsible for human and animal infections [4]. In Spain, the endemic presence of VL and CL has been reported for decades, with one of the Europe's highest incidence rates (0.4–0.7 annual cases per 100,000 population) [5]. Recent reports have also shown an increasing trend in human leishmaniosis cases in Spain [6].

In Spain, domestic dogs have predominantly maintained the enzootic cycle of *L. infantum*, with two widely distributed *Phlebotomus* species (*Ph. perniciosus* and *Ph. ariasi*) as competent vectors [7]. However, changes in the dynamics of *L. infantum* have occurred in the Iberian Peninsula in recent decades, resulting in sporadic human leishmaniosis outbreaks [8]. In fact, the largest outbreak of leishmaniosis ever reported in

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Europe occurred in Fuenlabrada (Madrid, central Spain) between 2009 and 2012, affecting over 800 people [9]. Several epidemiological investigations revealed that landscape changes favored interactions between humans and wild lagomorphs, which served as the relevant reservoirs [10]. High circulation of *L. infantum* in wild lagomorphs was later detected in other urban and peri-urban areas in Spain with a high incidence of human leishmaniosis [11].

Located in southern Spain, Andalusia is the most populated region in the country, which is also one of the four Spanish regions where 90% of the human leishmaniosis cases are reported [5,12,13]. The incidence of human leishmaniosis in Andalusia has been increasing in recent years, particularly in some health districts [13]. This epidemiological scenario reinforces the need to perform detailed epidemiological studies in hotspot areas with high incidence of human leishmaniosis, as well as to enhance the understanding of this zoonosis and advance in the implementation of effective preventive and control strategies. While *L. infantum* is recognized as a multi-host pathogen with significant public health implications, there is a paucity of scientific information concerning its epidemiology within a One Health approach. Previous studies have neither evaluated interactions between wild and domestic *Leishmania* spp. reservoirs nor fully elucidated the roles of human and vector populations in disease epidemiology [6,11]. Against this background, interdisciplinary studies that comprehensively assess the epidemiology of leishmaniosis in high-risk areas are encouraged [6]. Therefore, the aim of our study was to holistically assess the epidemiology of *L. infantum* in an area with a high incidence of human leishmaniosis and to evaluate both human exposure and protozoan circulation in domestic and wild reservoirs as well as in competent vectors.

Materials and methods

Study area

Our study area focused on the municipality of Castro del Río (37° 41′ 19″ N, 4° 28′ 54″ W) in the province of Cordoba (Andalusia, southern Spain) (Figure 1). This municipality is located at an altitude of 237 m above sea level and has a total population of 7,711 inhabitants and a population density of 35.5 people/km^2 [14].

Human leishmaniosis cases in our study area

This study was conducted in 2022, after four cases of VL in 2017 [1], 2019 [1], 2020 [1] and 2021 [1] were officially diagnosed in Castro del Río, including the death of one patient in 2021. This represents an accumulative incidence of 10.3 per 100,000 population between 2017 and 2021 (data provided by the Regional Government of Andalusia). This epidemiological information is supported by the high incidence of human leishmaniosis (0.81 per 100,000 population) reported in the same five-year period (2017–2021) in the health district to which Castro del Río belongs, which is twice the incidence of leishmaniosis reported in Andalusia (0.41 per 100,000 population) [13] (Figure 1).

Study design

The study area was divided into three zones based on their eco-epidemiological characteristics (Figures 1 and S1):

- Zone 1 (Z1): An urban and peri-urban area close to the river, with a large amount of organic matter due to agricultural activities. Note the high abundance of wild lagomorphs in this area (where hunting is prohibited) and in an adjacent hunting area.
- Zone 2 (Z2): The urban core has the highest population density and no agricultural or livestock activity. The mammal community is mainly composed of domestic animals. The central part of Z2 lies at a higher altitude (~40 m) than Z1 and Z3.
- Zone 3 (Z3): This area has several livestock farms interspersed with urban areas and agricultural landscapes.

Between August 2022 and April 2023, blood samples were collected from 145 healthy human blood donors. Blood and hair were also obtained from 41 domestic (n = 24) and stray/shelter (n = 17) randomly selected dogs living in the three zones studied. For lagomorph sampling, blood, spleen, and ear skin, whenever possible, were collected from 41 individuals (37 wild rabbits -*Oryctolagus cuniculus*- and 4 Iberian hares -*Lepus granatensis*-) randomly selected, taking advantage of hunting activity in the hunting area adjacent to Z1. An epidemiological questionnaire was also administered anonymously to obtain information on the individual characteristics and living habits of humans and dogs sampled in Z1-Z3 (Supplementary material). The geographical location of the sampled individuals is shown in Figure 2.

Collection and identification of sandflies

Between May 2022 and October 2022, sandflies were captured using CDC light traps (John W. Hock Company, Gainesville, USA) baited with CO_2. The traps were randomly positioned indoors and outdoors at eight georeferenced sampling sites distributed across the three zones specified (Figure 1). Sampling was carried out between sunset and sunrise at intervals of approximately 2–4 weeks. The captured sandflies were sorted, counted, and stored in absolute ethanol

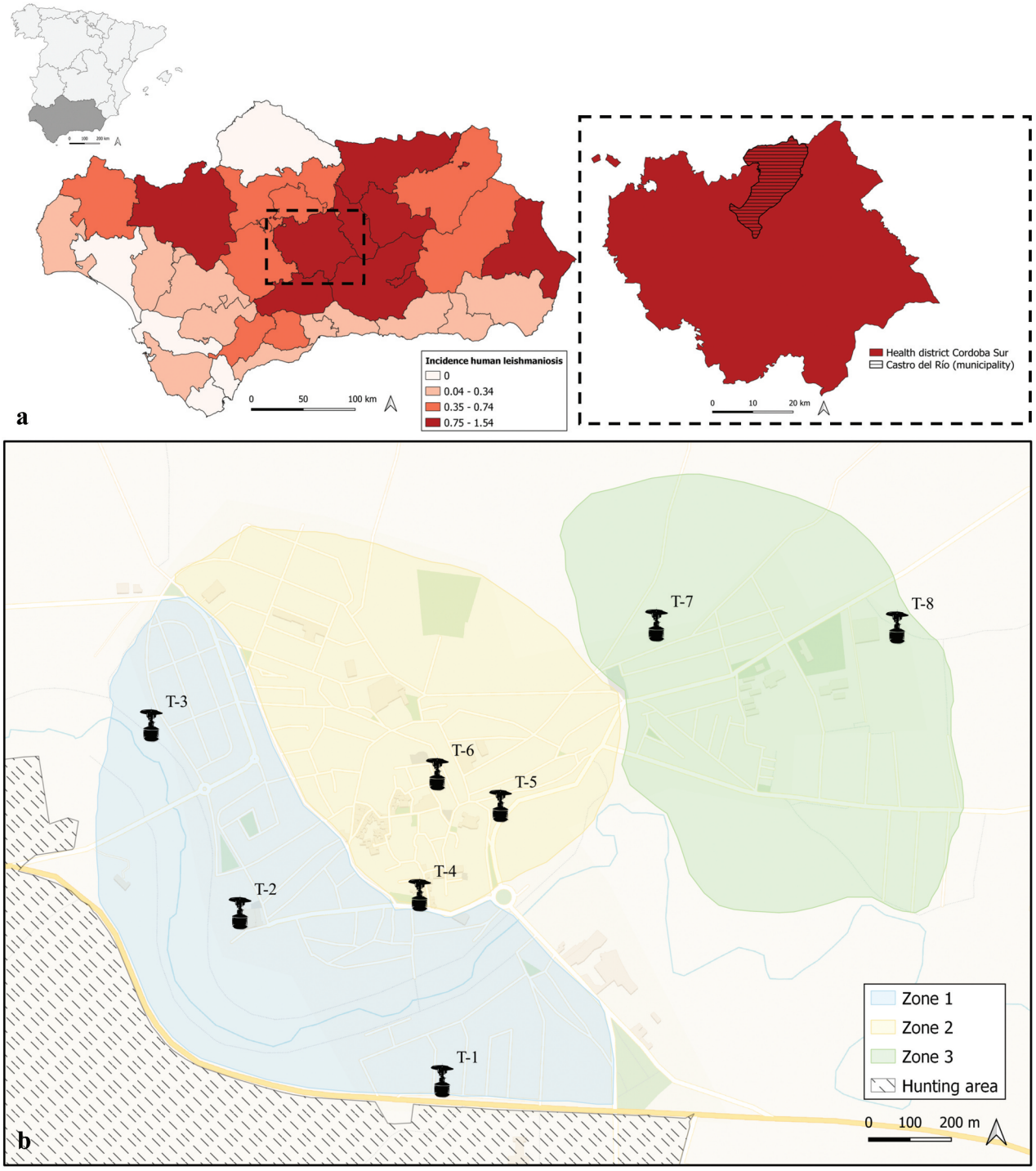

Figure 1. (a) Spatial representation of the incidence of human leishmaniosis in Andalusia (southern Spain) by health districts (official data from the regional Andalusian government), showing the location of the municipality of Castro del río in the Cordoba Sur district (inset). (b) Subdivisions (Z1-Z3) of the urban area of Castro del río according to ecological and environmental features are represented, including the spatial distribution of CDC light traps for sandflies. A hunting area with a high abundance of wild lagomorphs is shown close to Z1.

under freezing conditions (−20ºC). The specimens were then sexed and morphologically identified using identification keys [15].

Serological analysis

Serum samples were tested in duplicates for anti-*L. infantum* antibodies by the indirect fluorescent antibody test (IFAT) using slides coated with *L. infantum* promastigotes, as previously described [16]. A positive sample was determined as a cutoff titer of 1:80 for humans and dogs [17] and 1:50 for wild lagomorphs [16]. Positive sera were titrated in two-fold serial dilutions.

Molecular analysis

To extract DNA from competent *L. infantum* vectors (*Ph. perniciosus* and *Ph. ariasi*), DNA from engorged

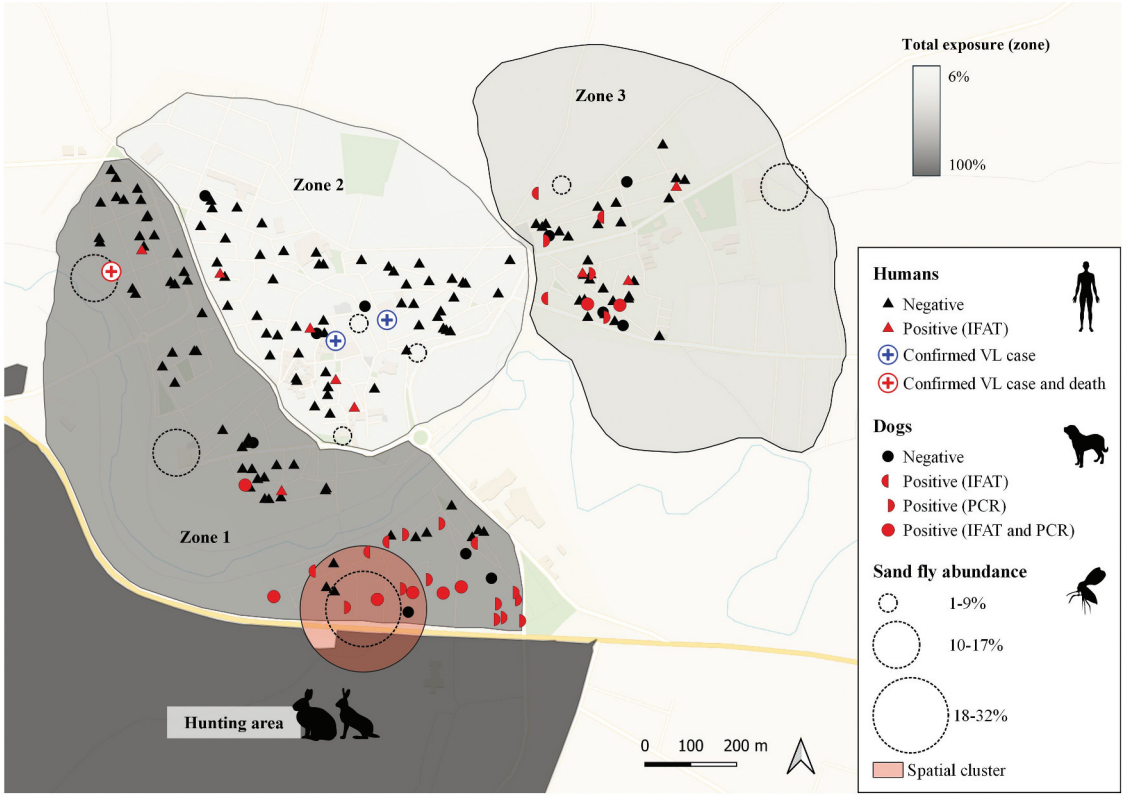

Figure 2. Spatial distribution of individuals sampled per zone in the municipality of Castro del Río (Andalusia, Spain), also showing *L. infantum* positivity (by IFAT and/or PCR) among sampled humans and dogs, and the abundance of sandflies collected at each sampling point. Total *L. infantum* exposure (IFAT and/or PCR) refers to the joint circulation of the protozoan in all hosts and competent vectors sampled in each evaluated area (Z1-Z3 and hunting are). The red circle denotes the significant spatial cluster of high *L. infantum* exposure observed in the study area ($p < 0.05$).

individuals was processed individually, while unfed females were pooled (five sandflies/pool). DNA extraction from sandflies was performed using the Quick DNA-RNA Pathogen kit (Zymo Research, Irvine, U.S.A.). Meanwhile, DNA from lagomorphs (spleen and skin) and dogs (hair) was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) and QIAamp Blood and Tissue Kit (QIAGEN, Hilden, Germany), respectively. To detect *L. infantum* DNA, quantitative PCR (qPCR) was performed to detect *L. infantum* kinetoplast DNA (kDNA) minicircles (about 10,000 copies), as previously described [18]. Parasite load was quantified using a standard curve generated in duplicate. The curve was constructed by serially diluting *L. infantum* DNA extracted from a known number of parasites (M/CAN/ES/97/10,445), ranging from 10^6 parasites/g to 0.01 parasites/g. Ct values below 37.6 were considered positive (detection limit: 0.1 parasite/g), and the number of parasites was calculated by interpolating Ct values to the standard curve [18].

L. infantum-positive samples with the lowest Ct values (≤28) were amplified by conventional PCR [19] and sent for DNA sequencing (Stab Vida, Caparica, Portugal). The data were screened using the BLASTn Tool (GenBank, Rockville, U.S.A.). A maximum likelihood tree (based on 5,000 replicates) was constructed using the Hasegawa-Kishino-Yano model, selected according to the lowest Bayesian Information Criterion (BIC) [20] and including a *Trypanosoma avium* sequence (accession number: AF027214) as outgroup.

Sandfly blood meal analysis

The engorged sandflies were categorized from 1 to 5, with 1 being fresh blood and 5 being the remains of digested blood in the abdomen [21]. Only individuals classified as 1 or 2 were selected for blood-feeding analysis. PCR amplification was performed, as previously described [22]. Sequencing results were evaluated as above.

Statistical analysis

Positivity was calculated as the ratio of the number of positive animals to the total number of individuals tested, using binomial confidence intervals (95%CI) [23]. Associations between the selected explanatory variables and *L. infantum* exposure were evaluated using Pearson's chi-square test or Fisher's exact test, as appropriate. A Generalized Linear Model (GLM) with a binomial error distribution and logit link function was constructed. All statistical analyses were performed using R software, version 4.1.3 [24]. Significant differences were considered with p-value ≤ 0.05.

The sandfly infection rate was estimated using the PooledInfRate v.4 statistical software package [25]. The Minimum Infection Rate (MIR) was calculated using the formula [number of positive pools/total specimens tested]. The Maximum Likelihood Estimate (MLE) was used for a more precise estimate as it does not assume that there is only one positive sandfly per positive pool [26]. Rates were expressed as the number of infected sandflies per 1000 specimens collected.

A spatial cluster analysis was applied using a Bernoulli model to detect significant clusters of high *L. infantum* exposure in humans, dogs, and vectors at the level of sandfly traps, using SaTScan v.10.1.2 software. The number of Monte Carlo simulations was set to 1,000 for the cluster scan statistic. SaTScan was used to estimate relative risk (RR), representing the relative frequency of cases (positive individuals to IFAT and/or qPCR) for each cluster. Clusters were considered significant at $p \leq 0.05$.

Results

Surveillance of L. infantum in human and animal hosts

Serological analysis revealed an overall seroprevalence of 6.2% (9/145; 95%CI: 2.3–10.1) among human donors (Table 1). The seropositive human cohort was predominantly male (66.7%; 6/9) and adult, with a mean age of 50.6 years (range: 30–66) (Table 2). At the spatial level, the highest *L. infantum* seroprevalence was reported in Z3 (9.1%; 3/33) (Table 1; Figure 2). No potential risk factors associated with *L. infantum* exposure were found in the human population included.

Among the dogs analyzed, the seroprevalence was 36.6% (15/41; 95%CI: 21.8–51.3) (Table 1). Molecular analysis of hair samples revealed a prevalence of 56.1% (23/41; 95%CI: 40.9–71.3), resulting in a total exposure (by IFAT and/or qPCR) of 73.2% (30/41; 95% CI: 59.6–86.7). The estimated parasite load showed considerable variability, ranging from 0.1 to 2,170 parasites/sample (median: 4.8). Notably, dogs showed the highest exposure in Z1 (84.0%; 21/25) (Table 1; Figure 2). The GLM model showed that stray/shelter dogs (94.1%; 16/17) were 20.8 times more exposed to *L. infantum* than domestic animals (58.3%; 14/24) ($p = 0.016$). Age was also identified as a risk factor with significantly ($p = 0.014$) higher exposure in dogs over 2 years old (85.2%; 23/27; OR: 10.7) compared to those less than 2 years old (50.0%; 7/14). Two of the 41 dogs showed clinical alterations (i.e. skin lesions) compatible with leishmaniosis during the sampling.

Among the wild lagomorphs, the serosurvey yielded a seroprevalence of 46.3% (19/41; 95%CI: 31.1–61.6) (Table 1). By molecular analysis, the prevalence was 50.0% (20/40; 95%CI: 34.5–65.5) in spleen samples, with an estimated parasite load of 6 to 17,316 parasites/g (median: 109), and 97.6% (40/41; 95%CI: 92.8–100) in ear skin samples, with an estimated parasite load of 321 to 40×10^6 parasites/g (median: 251,285). Notably, *L. infantum* exposure was 100% (41/41; 95%CI: 94.1–100) in lagomorphs. No clinical signs and lesions compatible with leishmaniosis were observed in the lagomorphs sampled.

Of the three positive wild lagomorphs selected for cPCR, two samples were sequenced and registered in GenBank (accession numbers: PP502926 and PP502927). Molecular analysis showed a strong homology (99.9–100%) between these isolates and other sequences obtained from animals and humans in southern Spain (Figure 3).

Table 1. Summary of surveillance results of *L. infantum* in human and animal hosts.

	Humans	Dogs	Wild lagomorphs
Serological results (IFAT)	6.2% (9/145; 95%CI: 2.3–10.1)	36.6% (15/41; 95%CI: 21.8–51.3)	46.3% (19/41; 95%CI: 31.1–61.6)
Antibody anti-*Leishmania* spp. titer (n)	1/80 ($n = 3$) 1/160 ($n = 5$) 1/320 ($n = 1$)	1/160 ($n = 8$) 1/320 ($n = 2$) 1/640 ($n = 1$) 1/1280 ($n = 2$) 1/2560 ($n = 1$) 1/5120 ($n = 1$)	1/50 ($n = 7$) 1/100 ($n = 10$) 1/400 ($n = 1$) 1/800 ($n = 1$)
Molecular results (qPCR) for *L. infantum* (type of samples)	–	Hair: 56.1% (23/41; 95%CI: 40.9–71.3)	Spleen: 50.0% (20/40; 95%CI: 34.5–65.5) Ear skin: 97.6% (40/41; 95%CI: 92.8–100)
Range of estimated *L. infantum* load (type of samples)	–	0.1–2,170 parasites/hair sample (median: 4.8)	Spleen: 6–17,316 parasites/g (median: 109) Ear skin: 32–40 × 10^6 parasites/g (median: 251,285)
Total exposure to *L. infantum* of individual analyzed (% positive IFAT and/or qPCR)	6.2% (9/145; 95%CI: 2.3–10.1)	73.2% (30/41; 95%CI: 59.6–86.7)	100% (41/41; 95%CI: 94.1–100)
Total exposure to *L. infantum* of individual analyzed (% positive IFAT and/or qPCR) by zones of the municipality	Z1 (3.6%; 2/56) Z2 (7.3%; 4/55) Z3 (9.1%; 3/33)	Z1 (84.0%; 21/25) Z2 (0.0%; 0/3) Z3 (66.7%; 8/12)	–

Table 2. Details of *L. infantum*-seropositive human individuals from Castro del río (CR) detected in the serosurvey.

ID	Sex	Age	Job	Residence in CR	Residence other than CR in last 2 years	History of clinical signs/ lesions leishmaniosis*	Dog owner	Exposure sandflies in period of activity**	Antibody titers
Case 1	Male	55	Baker	Zone 1	No	No	Yes	No	1/80
Case 2	Female	66	Retired	Zone 2	No	No	No	Yes	1/80
Case 3	Female	30	Caretaker	Zone 2	No	No	No	No	1/160
Case 4	Male	43	Builder	Zone 2	No	No	Yes	No	1/160
Case 5	Male	52	Plumber	Zone 2	No	No	Yes	Yes	1/80
Case 6	Male	47	Builder	Zone 3	No	No	No	Yes	1/160
Case 7	Male	49	Farmer	Zone 3	No	No	Yes	Yes	1/160
Case 8	Male	58	Mechanic	Zone 3	No	No	No	No	1/160
Case 9	Female	55	Housekeeper	Zone 1	No	No	No	Yes	1/320

*Clinical signs and lesions included: cutaneous lesions, severe weight loss, splenomegaly, hepatomegaly, anemia, and persistent fever of unknown origin.
**The period of sandfly activity was considered to be between 19.00 h (sunset) and 09.00 h (sunrise).

Entomological surveillance

A total of 1,347 sandflies were caught during the study period. Of note, 72.2% (156/216) of traps captured at least one sandfly specimen per trap/night (mean: 6.2; range: 1–81). Spatial distribution analysis revealed statistically significant differences ($p < 0.001$) in the overall abundance of sandflies, with the majority collected in Z1 (64.3%; 866/1347), followed by Z3 (20.7%; 279/1347) and Z2 (15.0%; 202/1347) (Table 2).

The results for abundance, frequency, and density are shown in Table 3. A higher abundance of *L. infantum* vectors (*Ph. perniciosus* and *Ph. ariasi*) was found in Z1 (35.3%; 476/1347) compared to Z3 (15.4%; 207/1347) and Z2 (8.3%; 112/1347). The sex ratio was evenly distributed (1: 1), with females accounting for 49.1% (662/1347) of the total captured specimens. While most females were categorized as unfed (77.9%; 516/662), a substantial proportion of engorged (15.6%; 103/662) and gravid (13.0%; 86/662) individuals were also collected. According to the temporal distribution, while sandflies were captured in all sampled weeks (range: 46–355), most of them (72.4%; 975/1347) were collected between weeks 27 and 36 (Figure 4).

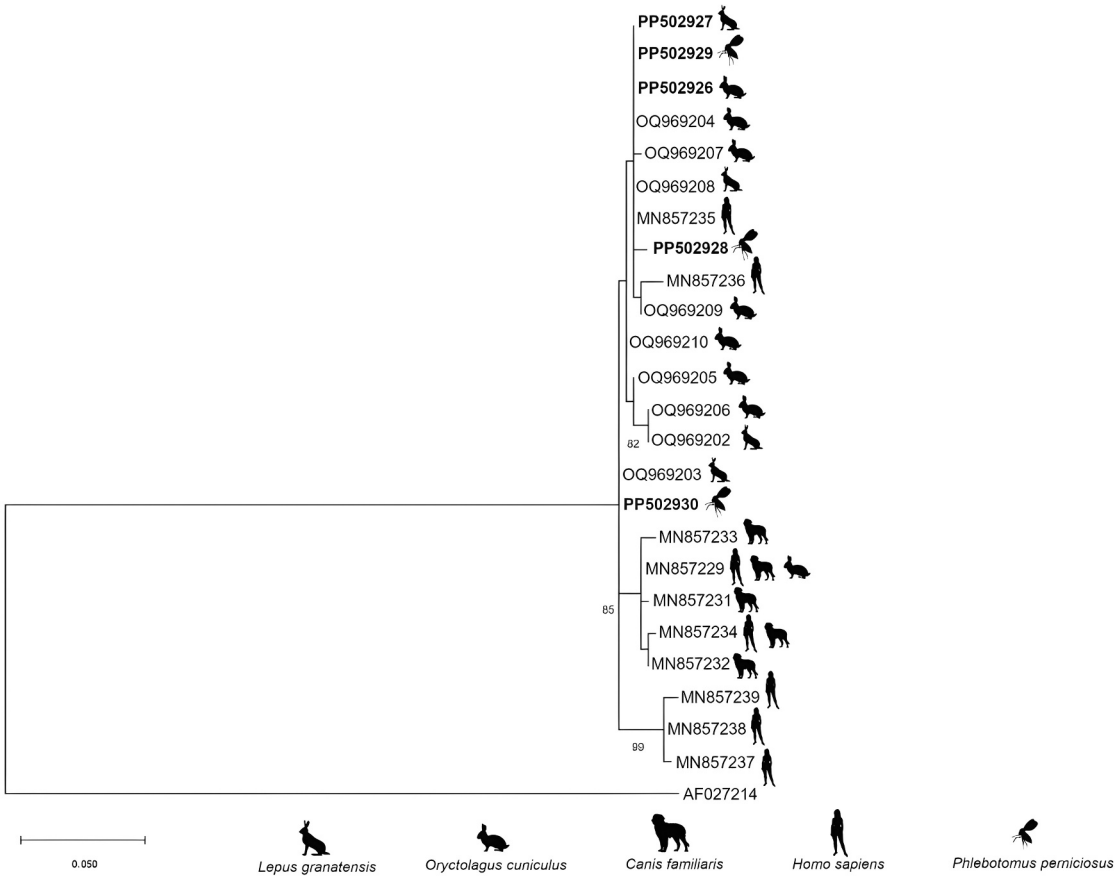

Figure 3. Phylogenetic relationship between *L. infantum* from wild lagomorphs and sandflies detected in this study and other *L. infantum* sequences from Spain available in the GenBank database. Sequences are presented with their accession numbers and represented with images of relevant species from infected hosts obtained from https://www.phylopic.org/. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates are collapsed.

Table 3. Details of sandfly species, according to zone and trap, collected in a study area with a high incidence of human leishmaniosis in Castro del Rio, Andalusia, Spain.

					Ph. perniciosus			*Ph. ariasi*			*Ph. papatasi*			*Ph. sergenti*			*Se. minuta*		
	Trap	Captured trap/total (%)	Sex	Total (N)	*A*	*D*	*F*	*A*	*D*	*F*	*A*	*D*	*F*	*A*	*D*	*F*	*A*	*D*	*F*
Zone 1	T-1	31.2	230♂ 190♀	**420**	41.7	6.5	66.7	2.1	0.3	25.9	0.7	0.1	11.1	0.7	0.1	7.4	54.8	8.4	77.8
	T-2	14.6	106♂ 91♀	**197**	55.8	4.1	63.0	1.5	0.1	11.1	0.5	0.0	3.7	3.1	0.2	22.2	39.1	2.9	51.9
	T-3	18.5	106♂ 143♀	**249**	70.3	6.5	77.8	1.6	0.2	14.8	0.8	0.1	7.4	0.0	0.0	0.0	27.3	2.5	59.3
	Total	**64.3**	**442♂ 424♀**	**866**	**53.1**	**5.7**	**69.1**	**1.9**	**0.2**	**17.3**	**0.7**	**0.1**	**7.4**	**1.0**	**0.1**	**9.9**	**43.3**	**4.6**	**63.0**
Zone 2	T-4	1.9	15♂ 10♀	**25**	68.0	0.6	29.6	4.0	0.0	3.7	0.0	0.0	0.0	12.0	0.1	11.1	16.0	0.2	14.8
	T-5	7.6	48♂ 54♀	**102**	53.9	2.0	74.1	2.0	0.1	7.4	10.7	0.4	33.3	2.0	0.1	7.4	31.4	1.2	59.3
	T-6	5.6	41♂ 34♀	**75**	46.7	1.3	44.4	2.7	0.0	7.4	0.0	0.0	0.0	5.3	0.2	14.8	45.3	1.3	48.2
	Total	**15.0**	**104♂ 98♀**	**202**	**53.0**	**1.3**	**49.4**	**2.5**	**0.1**	**6.2**	**5.5**	**0.1**	**11.1**	**4.5**	**0.1**	**11.1**	**34.7**	**0.9**	**40.7**
Zone 3	T-7	7.0	58♂ 36♀	**94**	61.7	2.12	66.7	1.1	0.0	3.7	4.3	0.2	11.1	1.1	0.0	3.7	31.9	1.1	40.7
	T-8	13.7	82♂ 103♀	**185**	80.0	5.5	70.4	0.0	0.0	0.0	2.7	0.2	14.8	1.1	0.0	7.4	16.2	1.1	51.9
	Total	**20.7**	**139♂ 140♀**	**279**	**23.8**	**3.8**	**68.5**	**0.4**	**0.0**	**1.9**	**1.0**	**0.2**	**13.0**	**1.1**	**0.0**	**5.6**	**6.9**	**1.1**	**46.3**
Total		100	1347	**1347**	57.4	3.6	61.6	1.6	0.1	9.3	1.9	0.1	10.2	1.6	0.1	9.3	37.5	2.3	50.5

A (Abundance) (% specimens of a given species/total sandflies); D (Density) (specimens/trap/night); F (Frequency) (% positive traps for a given species). Data were estimated.

In terms of blood-feeding preferences, unambiguous sequences were successfully obtained from six sandflies. These results highlight the diversity of blood sources, including small ruminants, cats, poultry, and humans in our study area.

Detection of *L. infantum* in sandflies

The prevalence of *L. infantum* in sandflies was 65.7% in the pools analyzed ($n = 70$) and 25.8% in individual sandfly specimens ($n = 31$). This gave an overall MIR of 138.1 (103.9–172.3) and an MLE of 196.50 (152.1–-250.2). The estimated parasite load in sandflies ranged from 0.1 to 383,418 parasites/sandfly (median: 1). Positivity to *L. infantum* was mainly found (88.9%; 48/54) between July and September. A statistically significant higher *L. infantum* positivity was found in sandflies from Z1 (75.9%; 44/58) compared to those collected in Z2 (31.3%; 5/16) and Z3 (18.5%; 5/27) ($p < 0.001$). Interestingly, in one engorged *Ph. perniciosus*, human and *L. infantum* DNA were detected simultaneously.

Molecular analysis showed a high homology (99.9-–100%) between the sandfly sequences obtained (accession numbers: PP502928–PP502930) and the wild lagomorph isolates detected in the present study as well as with other sequences of *L. infantum* from animals (mainly wild lagomorphs) and humans in southern Spain (Figure 3).

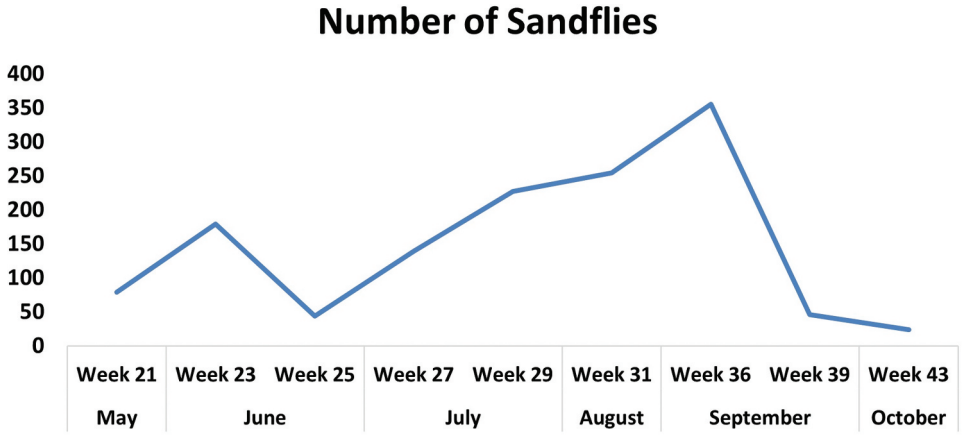

Figure 4. Seasonal dynamics of sandfly activity (by month) in 2022 in Castro del Rio (Andalusia, southern Spain), based on the total caught in eight traps placed in zones 1 ($n = 3$), 2 ($n = 3$), and 3 ($n = 2$).

Spatial clustering in hosts and sandflies

Spatial analysis showed that the distribution of exposure to *L. infantum* among humans, dogs, and sandflies in the urban zones (Z1-Z3) was heterogeneous. Total *L. infantum* exposure was significantly higher in Z1 (48.2%; 67/139) compared to Z3 (22.2%; 16/72) and Z2 (12.2%; 9/74) ($p < 0.001$). Furthermore, a significant spatial cluster was detected in Z1 (37.684858 N, 4.480886 W) for exposure to *L. infantum* in humans, dogs, and vectors (RR: 2.1; $p < 0.001$) (Figure 2).

Discussion

Complex multi-host systems involving interactions between humans, sandflies, domestic animals, and wild reservoirs drive endemic circulation of *Leishmania* spp. Therefore, the development of effective control, surveillance, and prevention measures against leishmaniosis requires studies using an integrated One Health approach, particularly in high-risk regions [6]. Our study demonstrated a high abundance and diversity of sandfly species, as well as high exposure to *L. infantum* in vectors, dogs, and lagomorphs, favoring human cases in an area with a high incidence of leishmaniosis. This study provides a representative framework for any population living in endemic regions of *L. infantum* circulation with a meso-Mediterranean climate and a similar epidemiological scenario.

In our study area, human seroprevalence was found to be 6.2%, which is within the range of values previously reported (5.1–12.9%) in different areas of a neighboring province with a high incidence of human leishmaniosis in Andalusia [17], one of the four regions in Spain where 90% of the human leishmaniosis cases are reported [5,12,13]. The incidence of human leishmaniosis in our study area was unusually high compared to that reported in Andalusia between 2017 and 2021 [13]. Our results indicate a moderate exposure to *L. infantum* in the asymptomatic human population in this Mediterranean hotspot of human leishmaniosis, which is higher than that detected in several highly endemic areas in eastern Spain (1.0–-3.1%) [27,28] and other endemic European countries, such as Portugal (4.8%) and Italy (4.9%) [29,30]. Therefore, in areas with a high incidence of human leishmaniosis, the screening of blood donors could be a possible surveillance strategy to overcome the underdiagnosis of human leishmaniosis [30].

Our study area is also considered a hyperendemic zone for *L. infantum* circulation in dogs [31]. In this context, the overall seroprevalence (36.6%) observed was higher than that reported in previous studies (9.3-–30.0%) carried out in southern Spain [31]. A high prevalence (56.1%) was also found in the study area compared to other reports in the same or neighboring regions (44.0–49.9%) [32,33]. The overall exposure detected in dogs in the present study (73.2%) reinforces the important role of this species as a major reservoir in the domestic cycle of *L. infantum* in certain microfoci of the disease [11]. The multivariate model showed that exposure to *L. infantum* was higher in stray/shelter dogs (94.1%) than in domestic dogs (58.3%). This finding is consistent with the outdoor lifestyle of stray dogs and their high exposure to sandfly bites [34]. Age was also identified as a risk factor, with a significantly higher prevalence in older dogs, which is also supported by increased contact with infected sandflies over time [35]. Our results highlight the need to implement effective preventive measures against sandflies in dogs, especially older animals and those with outdoor habits, to reduce the risk of canine and human leishmaniosis in hyperendemic areas [35].

Previous surveys have also discussed the significant implications of wild reservoirs, wild lagomorphs in particular, in the epidemiological cycle of *L. infantum*. The wild lagomorphs in our study showed a higher exposure to *L. infantum* (100%) than those found in the same species (23.7–78.2%) during the largest outbreak of human leishmaniosis ever reported in Europe [36] and was much higher than those observed in other regions of Spain, such as Valencia (0.6%) and Catalonia (12.0%) [37], and in other countries in southern Europe, such as Italy or Greece (6.5–7.5%) [38,39]. Furthermore, the high circulation of *L. infantum* in the wild lagomorph population analyzed overlaps with other areas with a high incidence of human leishmaniosis in southern Spain [13,40]. Castro del Río is also located within the spatial cluster of significantly higher circulation of this protozoan in wild lagomorphs, as recently reported [40]. In addition, in our study municipality, Z1 showed the highest exposure to *L. infantum* compared to the other two zones (Z2 and Z3), which also registered a significant spatial cluster of high exposure to this parasite within our study area. In particular, the proximity of Z1 to a hunting area with wild lagomorphs showing 100% exposure to *L. infantum* indicates the importance of lagomorphs as a potential source of *L. infantum* infection for sandflies and thus their central epidemiological role in the maintenance and spread of this protozoan parasite [11].

The abundance and diversity of sandfly species found in the present study are consistent with those previously observed in urban and rural areas of Spain [41]. Surveillance of *L. infantum* in sandflies revealed a high presence of parasite DNA (53.5%, 54/101) in the study area, like values reported in other active leishmaniosis foci in the Mediterranean basin (51.9%) [42], although lower than those described in other human outbreaks in Spain (>58.5%) [41]. *L. infantum* transmission is often associated with specific microfoci in urban and rural areas [43], which are often linked to microenvironmental factors that favor

an increase in sandfly abundance and consequently the risk of parasite transmission [11]. It is noteworthy that Z1 had both a significantly higher vector abundance and *L. infantum* prevalence than the other two zones sampled; it also has larger plots and abundant vegetation, which may be positively correlated with the abundance of *L. infantum* vectors in residential areas [44]. In the study area, a bimodal pattern associated with the abundance of *Ph. perniciosus* was observed, with peaks in July and September, like those previously reported in studies carried out in the Mediterranean area [45]. Furthermore, different host species were detected as blood-feeding sources of sandflies, suggesting opportunistic feeding behavior in *L. infantum* vectors [45]. Interestingly, a *L. infantum*-positive *Ph. perniciosus* feeding on a human was detected, highlighting the potential zoonotic circulation of the parasite in the study area and consequently its potential impact on public health.

Given these findings, it is crucial to identify microhabitats with a higher risk of *L. infantum* circulation to implement targeted surveillance and integrated prevention and control measures for both animal reservoirs and competent vectors. A comprehensive approach to environmental vector management should be prioritized, including the removal of tree stumps, filling soil gaps and indoor cracks/crevices to prevent oviposition and emergence of adult sandflies, regular cleaning of peridomicile areas and animal shelters, and proper disposal of household and organic waste [46]. The use of fine-mesh mosquito nets (0.3 mm^2 light size) in high-risk areas, along with disinsection programs targeting both adults and immature stages, is recommended [13]. These programs should incorporate indoor residual spraying, insecticide-treated bed nets, treated bed linen and clothing, durable wall linings, and space spray applications [33,46]. These measures have demonstrated efficacy in reducing the sandfly abundance and should be considered essential components of vector control strategies in the study area [9,13].

In addition to vector control, the role of wild lagomorphs as key reservoirs of *L. infantum* must be addressed [9]. Surveillance programs focused on monitoring parasite circulation in these hosts are essential to understand transmission dynamics. Furthermore, population control strategies should be considered in areas where the prevalence of the parasite in these reservoirs poses an imminent problem for public health, including measures to reduce the abundance of wild lagomorphs in high-risk areas and the controlled removal of their breeding sites, particularly those in proximity to urban and peri-urban areas [47]. These actions can significantly reduce the risk of human exposure to the parasite as evidenced in the leishmaniosis outbreak in Madrid, where these animal species also played a significant role like reservoirs [9,47].

For canine reservoirs, a multimodal approach is recommended to mitigate the risk of transmission. The use of topical repellent, such as permethrin spot-on formulations (short duration: 3–4 weeks) or deltamethrin-impregnated collars (long duration: 12 months), can effectively reduce sandfly bites and, consequently, the infection rates [48,49]. In addition, vaccination against *L. infantum* should be promoted to prevent the infectiousness of dogs to sandflies and to reduce the likelihood of clinical manifestations in infected animals [48,50]. Regular veterinary checkups, particularly after the season of high vector activity, are strongly advised in endemic areas to detect and manage early infections.

In the human population, raising awareness about leishmaniosis is paramount. Public health initiatives should focus on educating communities about the disease [51], including transmission cycle, key reservoirs, and effective personal protection measures. The use of protective clothing that minimizes exposed skin and the application of approved insect repellents are encouraged, especially during periods of high sandfly activity. Additionally, targeted interventions for at-risk populations, such as immunocompromised individuals, should be reinforced to mitigate severe disease outcomes [52,53].

Therefore, a multidisciplinary and One Health approach integrating vector control, prevention of infection in domestic and wild reservoirs, and human awareness is essential to effectively mitigate the transmission of *L. infantum* and reduce its impact on both animal and human populations.

Conclusions

The high *L. infantum* exposure found in wild lagomorphs as well as the strong homology between sequences found in these species and those obtained from sandflies strongly support that wild lagomorphs play a key role as reservoirs of *L. infantum*, thus favoring the occurrence of human leishmaniosis and high prevalences in dogs at the wildlife-human-domestic interface. This epidemiological network is enhanced by the abundant presence of sandflies, mainly *Ph. perniciosus*, in microhabitats that constitute spatial clusters generally associated with high *L. infantum* circulation. This study emphasizes the relevance of using a One Health approach to conduct interdisciplinary studies on leishmaniosis in order to develop integrated prevention and control strategies in high-risk areas.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Ethical statement

All protocols, amendments, and procedures were conducted in accordance with the guidelines approved by the Regional Government of Andalusia, in compliance with the provisions of current legislation. In particular, these procedures comply with Law 32/2007 concerning the welfare of animals during their exploitation, transport, and experimentation. This law has been amended by Law 6/2013 and Royal Decree 53/2013, which establishes the basic rules for the protection of animals used in experiments and other scientific purposes, including teaching. The study protocol received approval from the Ethics Committee of the Regional Authority (Ref. 5509), and informed consent was obtained from all participants and dog owners prior to inclusion in this study. The lagomorphs were legally hunted by licensed hunters during the hunting season in accordance with Spanish and EU legislation. This process did not involve the deliberate killing of animals, and blood samples were not collected specifically for this study. Therefore, no ethical approval was required for this species.

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