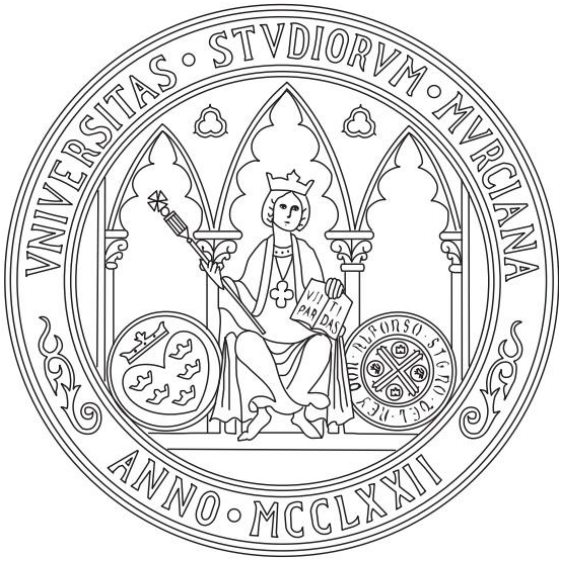

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Advances in the epidemiology of leishmaniosis in animals and humans: genetic diversity of *Leishmania infantum*, exposure to *Phlebotomus perniciosus* vector and coinfections with other pathogens

Avances en la epidemiología de la leishmaniosis en animales y humanos: diversidad genética de *Leishmania infantum*, exposición al vector *Phlebotomus perniciosus* y coinfecciones con otros patógenos

Dña. María Ortuño Gil

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Tesis Doctoral

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Memoria presentada por la Graduada en Veterinaria

María Ortuño Gil

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La presentación de la Tesis Doctoral titulada "Advances in the epidemiology of leishmaniosis in animals and humans: genetic diversity of *Leishmania infantum*, exposure to *Phlebotomus perniciosus* vector and coinfections with other pathogens" (Avances en la epidemiología de la leishmaniosis en animales y humanos: diversidad genética de *Leishmania infantum*, exposición al vector *Phlebotomus perniciosus* y coinfecciones con otros patógenos), realizada por María Ortuño Gil, bajo nuestra supervisión y dirección, para la obtención del grado de Doctor por la Universidad de Murcia.

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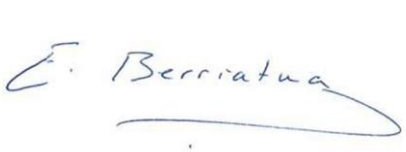

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A mi familia y amigos

“Cada día sabemos más y entendemos menos”

Albert Einstein

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Summary

In this PhD dissertation we have investigated several epidemiological aspects of phlebotomine sandfly- and tick-borne infections in dogs, cats, wildlife and humans in southern Spain. Specifically, we investigated: (1) the aetiology, prevalence and risk factors of infection with the flagellate, protozoan parasite *Leishmania infantum* (family Trypanosomatidae) in animals and humans, (2) the aetiology, prevalence and risk factors of infection with the sporozoan, protozoan parasites *Hepatozoon* spp. (family Hepatozoidae) and *Babesia* spp. (family Babesiidae) in animals, (3) the antibody prevalence of Toscana (TOSV) and Sicilia (SFSV) phleboviruses (family Phenuiviridae) in human blood donors, (4) the risk of coinfections with these pathogens in animals and humans, (5) the antibody response of blood donors to salivary antigens of *Phlebotomus perniciosus* sand fly vectors and (6) the relationship between *L. infantum* and TOSV infections and the antibody optical density against *P. perniciosus* salivary antigens.

Leishmania spp. infect reticular endothelial cells causing leishmaniosis in humans and animals, a potentially deadly disease. They are distributed in the Mediterranean basin including Spain where *L. infantum* is the only endemic species. The parasites are transmitted by the bite of infected female phlebotomine sandflies, and the vector species in Spain are *P. perniciosus* and *P. ariasi.* Human leishmaniosis (HumL) incidence in Europe is comparatively low, although it is underreported. The domestic dog (*Canis lupus familiaris*) is the primary reservoir of infection in the domestic environment, and it is estimated that 2.5 million dogs in Europe are infected and at risk of developing canine leishmaniosis (CanL). High prevalences of infection are reported in southern European countries, although only a relatively small proportion of infected dogs, unable to elaborate an effective immune response, develop clinical CanL. Leishmaniosis is considered an emergent disease because of climate change, tourism, migration and relocation of infected dogs. The distribution of *L. infantum* and its vectors is widening, and CanL is increasingly reported in previously non-endemic areas in Europe. There are several factors hindering the control of leishmaniosis: (i) it is a vector-borne disease, and vector population control is difficult because they breed in a wide range of terrestrial sites not readily amenable to insecticide treatment or other forms of control; (ii) factors influencing vector distribution and thus, human and canine exposure to infection are not well defined; (iii) leishmaniosis control is strongly based on the use of preventive insecticide treatments in dogs and they are only partially efficacious; (iv) it is a chronic infection with a high proportion of asymptomatically infected dogs that may transmit the parasite to the vector; (v) treatment

of CanL does not provide parasitological cure and relapses are frequent; (vi) there are no fully effective vaccines for dogs and no vaccine has been developed for humans and (vii) a wide range of domestic and sylvatic animal species could act as reservoirs of *Leishmania* spp. infection. Moreover, coinfections with other viral, bacterial or protozoan agents might influence host's susceptibility to leishmaniosis and thus, have an impact on its epidemiology and control, although information in this respect is scarce. Among the latter we consider sandfly-borne phleboviruses and tick-borne *Hepatozoon* and *Babesia* parasites of blood cells, as potential candidates influencing susceptibility to *L. infantum* infection. Phleboviruses are RNA stranded viruses widely prevalent in sand fly endemic areas. In Spain, up to seven different *Phlebovirus* species circulate among humans, animals and sandflies, including TOSV, SFSV, Naples, Granada, Massilia, Arbia and Arrabida phleboviruses. Infections are usually asymptomatically or mild, causing flu-like symptoms, although TOSV is responsible for sporadic cases of severe neurological infections. The main reservoir of these viruses is unknown and this is an important epidemiological and control limitation. Moreover, the implications of phleboviruses and *L. infantum* coinfection are poorly understood and so far, the common sandfly vector is the only epidemiological link between them.

Hepatozoon spp. and *Babesia* spp. comprise several species, most of which are host-species specific. They target leukocytes and erythrocytes, respectively, and whilst subclinical infections are common, they may cause severe disease and high mortality. Coinfections of *Hepatozoon* and *Babesia* parasites with *L. infantum* have been reported in domestic and wild animals but the pathogenic synergism between them remains unknown.

The contribution of this doctoral thesis to the knowledge of the epidemiology of these pathogens is presented in four chapters. In **Chapter 1,** stray cats and abandoned dogs from Murcia Region were clinically examined and analysed for *L. infantum* infection in blood, skin and lymphoid tissue samples and for *Babesia* spp. and *Hepatozoon* spp. infection in blood samples. We used a TaqMan probe, real-time Polymerase Chain Reaction (rtPCR) targeting kinetoplast DNA (kDNA) to detect *L. infantum* infection. A conventional, end-point PCR (cPCR) for 18S ribosomal RNA coding sequences was firstly used to screen for apicomplexan DNA with generic primers, and positive samples were subjected to genus-specific cPCR protocols. *Babesia* spp. and *Hepatozoon* spp. amplicons were sequenced and phylogenetic trees were constructed. A semi-quantitative estimation of *L. infantum* DNA was obtained considering the rtPCR threshold cycles (CTs), which is the inversely related

to the amount of target DNA sequences. The degree of agreement between *L. infantum* test results in different biological samples was analysed using *kappa* coefficient.

Clinical signs were detected in 53% (112/212) of cats and in 2% (2/82) of dogs. *Leishmania infantum* prevalences in dogs and cats were 44% (36/82) and 21% (44/212), respectively. *Hepatozoon felis* was detected in 25% (31/123) of cats and *Hepatozoon canis* in 13% (11/82) of dogs. Only one dog was infected with *Babesia spp*., identified as *B. vogeli*. Sequence analysis of *Hepatozoon* spp. revealed three different *H. canis* isolates and three *H. felis* isolates. Mixed *L. infantum* and *H. canis* or *H. felis* infections were detected in 7% of dogs and 5% of cats, respectively, but there was no significant statistical association between infection with the flagellate and the sporozoan parasites. The prevalence of *L. infantum* differed according to sample type, collection season and cat's clinical status. It was higher in lymphoid tissue and skin compared to blood in both dogs and cats, except in symptomatic cats in which it was similar. Moreover, prevalence in lymphoid tissue was higher and rtPCR CTs lower (indicating higher parasite load) in dogs compared to cats, possibly supporting cats' lower risk of developing systemic leishmaniosis than dogs and lesions being most commonly cutaneous. The most frequent feline leishmaniosis-compatible clinical signs in cats in the present study were cutaneous lesions and lymphadenomegaly. However, clinical signs were not associated to *L. infantum* infection. Also, the prevalence of *L. infantum* infection in dogs and cats was highest in winter, which is compatible with the long incubation period of *L. infantum* following a typical May to October transmission season in Murcia Region. Results from this study are important from a clinical and diagnostic perspective and would support a relevant role of cats in the epidemiology of *L. infantum.*

Chapter 2 builds on the previous study by investigating the prevalence of *L. infantum*, *Hepatozoon* spp. and *Babesia* spp. in wild carnivores, including foxes, badgers, beech martens, hedgehogs, wild cats, Egyptian mongooses, otters, genets and racoons. Animals came from Murcia, Comunidad Valenciana, Andalucía and Extremadura regions in southern Spain. DNA was extracted from spleen samples and *L. infantum, Hepatozoon* spp. and *Babesia* spp. DNA was amplified with the same PCR protocols used for dogs and cats, described in Chapter 1. The overall prevalences for *L. infantum, Babesia* spp. and *Hepatozoon* spp. in wildlife were 23%, 48% and 68%, respectively. Moreover, generic cPCR allowed the unexpected identification of *Cytauxzoon* spp. in one wildcat and *Sarcocystis* spp. in one genet. Sequence analysis revealed three different *Babesia vulpes*

isolates in foxes, three *Babesia* sp. badger type A isolates in badgers, 15 *H. canis* isolates in foxes and beech martes, three *H. felis* isolates in wildcats, and six *H. martis* isolates in beech martes, badgers and wildcats. It is the first report of *H. canis* in beech martens and that of *H. martis* in wildcats. Some of the *H. canis* and *H. felis* isolates were identical to those found in domestic animals from the same region (Chapter 1), suggesting a common transmission cycle for these parasites as also suggested for *L. infantum* in the study presented in Chapter 4. *Leishmania infantum* infection prevalence varied according to the study area, the year and the host species. It was significantly more prevalent in the southeast of Spain (Murcia and Comunidad Valencia) compared to the south and southwest (Andalucía and Extremadura) in foxes and badgers, and in 2013-2015 compared to 2016-2018 in foxes. In contrast to dogs and cats (Chapter 1), *L. infantum* infection was significantly, positively associated to *Hepatozoon* spp. and *Babesia* spp. infections, suggesting that being infected with the apicomplexan constitutes a risk factor for becoming infected with *L. infantum*, and vice versa. Further studies are required to assess this relationship in other populations and to clarify the possible clinicopathological mechanisms associated to these coinfections. Moreover, the findings from this study contribute to a better understanding of the aetiology and transmission cycles of *Hepatozoon* spp. and *Babesia* spp.

Chapter 3 focusses exclusively on human blood donors and the aim was to estimate the prevalence of *L. infantum* and sand fly-borne TOSV and SFSV, assess exposure to *P. perniciosus* sandfly bites and investigate the relationship between these infections and with exposure to the vector. The latter was done by analysing the donor's antibody response to *P. perniciosus* salivary gland homogenate (SGH) and to the recombinant rSP03B salivary protein. This was the first time that the humoral response to this vector species had been analysed in humans. We collected 670 blood samples from 575 blood donors from 13 rural and periurban towns and villages in Murcia Region during two blood donation campaigns in January-March 2017 and September-November 2018. Enzyme-linked immunosorbent assays (ELISA) were used to analyse antibodies against *P. perniciosus* salivary antigens with two in-house ELISA protocols and for *L. infantum* using a commercially available test. *Leishmania* DNA was analysed with the previously described rtPCR for kDNA. Virus neutralization tests were employed to analyse antibodies against TOSV and SFSV. Antibodies against SGH and/or rSP03B were detected in 98% of donors, but the correlation between antibody optical densities for these antigen preparations was only moderate (ρ =

0.39). Therefore, it would seem that the recombinant protein rSP03B is not an adequate marker for assessing *P. perniciosus* exposure. TOSV seroprevalence was 26%, whereas none of the donors where seropositive to SFSV. *Leishmania infantum* ELISA and rtPCR prevalences were 1% and 2%, respectively, and the overall *L. infantum* prevalence taking into account results from both techniques was 3%. Only 1% of donors tested positive to both TOSV and *L. infantum*. Antibodies against salivary gland homogenate were significantly higher in donors positive to TOSV and *L. infantum*. Multivariable regression models indicated that the risk for TOSV infection was greatest among older people living in rural areas and sampled in 2017. On the contrary, *L. infantum* prevalence was not associated to any demographic variable except that it was also greater in 2017 compared to 2018. Similarly, salivary antibodies were also significantly higher in 2017. TOSV seropositives were present in every blood donation centre with variable prevalence ranging from 10% to 44% whilst *L. infantum* positives were detected in six of thirteen blood donation centres and prevalences ranged between 3% to 10%. These results highlight major differences in the risk of TOSV and *L. infantum* infection between areas and years in Murcia Region, suggesting that transmissions of these pathogens are not dependent on each other and is subjected to marked interannual differences. Further studies are required to investigate the environmental and anthropic factors associated to TOSV and *L. infantum* distributions.

In **Chapter 4** we analysed genetic diversity between *L. infantum* strains from dogs, wildlife and humans. The latter included samples from human visceral leishmaniosis (VL) and cutaneous leishmaniosis (CL) cases and from asymptomatic blood donors. Animal samples came from infected, asymptomatic wildlife and from dogs with CanL following *in vitro* parasite culture of *L. infantum* isolates. Two genetic typing techniques were used, PCR-sequencing and Single Nucleotide Polymorphisms (SNPs) detection of kDNA and ribosomal Internal Transcribed Spacer (ITS)-2 sequences, and kDNA-PCR-Restriction Fragment Length Polymorphism (RFLP) with a panel of nine endonucleases. Analysis of ITS-2 sequences revealed *L. infantum* in all samples except for one Latin-American patient positive to *L. panamensis*. Two *L. infantum* ITS-2 positive strains differing in only one nucleotide were identified. In contrast, analysis of kDNA sequences allowed the classification of *L. infantum* into 11 different "SNP-genotypes" presenting 1-14 SNPs. Some SNP-genotypes were specific of humans or dogs whereas others were shared between dogs, humans and wildlife. Moreover, one dog was coinfected with two different SNP-

genotypes, which possibly indicates coinfection with different *L. infantum* strains. Genetic diversity analysed by PCR-RFLP was comparatively lower and it revealed only four different genotypes (B, F, S, T). Genotype B was the most frequent and genotypes S and T had not been previously reported. The phylogenetic analyses grouped the SNP-genotypes into three clusters, two of them conformed by human strains only and the other grouped strains from humans, domestic and wild animals. No association was found between specific genotypes and the leishmaniosis clinical presentation in humans. Genetic diversity was higher in symptomatic than asymptomatic individuals, and in humans compared to animals. These results confirm kDNA as a suitable marker for investigating genetic polymorphism, and that there is a wide genetic heterogeneity in *L. infantum* populations from south-eastern Spain including common and host-specific strains that suggest independent and shared transmission cycles between animals and humans, and the domestic and sylvatic environments.

All together the work carried out for this doctoral thesis contributes to our understanding of several aspects of the epidemiology of sandfly and tick-borne pathogens in humans, domestic and wild animals in southern Spain and other areas where these pathogens are present. We emphasize the importance of a global view of vector-borne infections, taking a One Health approach to the investigation of *L. infantum* epidemiology, targeting both humans and animals and analysing exposure to the main *L. infantum* vector in Western Europe. We report a high prevalence of infection with *L. infantum*, *Hepatozoon* spp. *Babesia* spp. and TOSV and provide evidence of shared transmission cycles between humans, domestic animals and wildlife. We hope that the information generated will contribute towards designing evidence-based control programs to reduce the impact of these pathogens on the human and animal population.

Resumen

En esta tesis doctoral, hemos investigado varios aspectos epidemiológicos de infecciones transmitidas por flebotomos y garrapatas en perros, gatos, fauna silvestre y humanos del sur de España. En concreto, hemos investigado: (1) la etiología, prevalencia y factores de riesgo de la infección por el protozoo flagelado *Leishmania infantum* (familia Trypanosomatidae) en animales y humanos, (2) la etiología, prevalencia y factores de riesgo de la infección por los protozoos esporozoarios *Hepatozoon* spp. (familia Hepatozoidae) y *Babesia* spp. (familia Babesiidae) en animales, (3) la prevalencia de anticuerpos frente a los *Phlebovirus* (familia Phenuiviridae) Toscana (TOSV) y Sicilia (SFSV) en personas donantes de sangre, (4) el riesgo de coinfecciones con estos patógenos en animales y humanos, (5) la respuesta de anticuerpos de los hemodonantes a los antígenos salivares de los vectores flebotomos *Phlebotomus perniciosus* y (6) la relación entre las infecciones por *L. infantum* y TOSV y la densidad óptica de anticuerpos frente a los antígenos salivares de *P. perniciosus.*

Leishmania spp. infectan células del sistema reticuloendotelial causando la leishmaniosis en humanos y animales, la cual es una enfermedad potencialmente mortal. Son endémicas en la cuenca Mediterránea incluyendo España, donde *L. infantum* es la única especie endémica. Los parásitos son transmitidos por la picadura de hembras de flebotomo infectadas, y las especies vectoras en España son *P. perniciosus* y *P. ariasi.* La incidencia de leishmaniosis humana (LHum) en Europa es comparativamente baja, aunque muchos casos no son registrados. El perro doméstico (*Canis lupus familiaris*) es el reservorio primario de la infección en el ambiente doméstico, y se estima que 2.5 millones de perros en Europa están infectados y, por ello, en riesgo de desarrollar la leishmaniosis canina (LCan). Se han reportado altas prevalencias de infección en los países del sur de Europa, aunque solo una relativamente pequeña proporción de los perros infectados, incapaces de elaborar una respuesta inmune efectiva, desarrollan la enfermedad. La leishmaniosis es considerada una enfermedad emergente debido al cambio climático, el turismo, la inmigración y el traslado de perros infectados. La distribución de *L. infantum* y sus vectores se está expandiendo, y la LCan está siendo cada vez más declarada en áreas de Europa previamente libres. Hay varios factores que dificultan el control de la leishmaniosis: (i) es una enfermedad vectorial, y el control de las poblaciones de vectores es difícil porque crían en un amplio rango de lugares terrestres en los que es complicado el tratamiento insecticida u otra formas de control; (ii) los factores que influyen en la distribución del vector y, por ello, en la exposición humana y canina a la infección, no están bien definidos; (iii) el control

de la leishmaniosis está fuertemente basado en el uso de tratamientos insecticidas preventivos en perros y sólo son parcialmente eficaces; (iv) es una infección crónica con una alta proporción de perros infectados asintomáticamente que pueden transmitir el parásito al vector; (v) el tratamiento de la LCan no produce una cura parasitológica y las recidivas son frecuentes; (vi) no hay vacunas plenamente efectivas para los perros y todavía no se ha desarrollado ninguna vacuna para humanos y (vii) existe un amplio rango de especies animales domésticas y silvestres que podrían actuar como reservorios de la infección por *Leishmania* spp. Además, las coinfecciones con otros agentes virales, bacterianos o protozoarios podrían influenciar la susceptibilidad del hospedador a la leishmaniosis y, por tanto, tener un impacto en su epidemiología y control, aunque la información al respecto es escasa. Entre éstos últimos consideramos los flebovirus transmitidos por flebotomos y los parásitos de células sanguíneas transmitidos por garrapatas *Hepatozoon* spp. y *Babesia* spp., como potenciales candidatos a influenciar la susceptibilidad a la infección por *L. infantum.*

Los flebovirus son virus de cadena de ARN ampliamente prevalentes en áreas endémicas de flebotomos. En España, hay hasta siete especies diferentes de *Phlebovirus* circulando entre humanos, animales y/o flebotomos, incluyendo los flebovirus TOSV, SFSV, Nápoles, Granada, Massilia, Arbia y Arrabida. Las infecciones son normalmente asintomáticas o leves causando síntomas parecidos a la gripe, aunque TOSV es el responsable de casos esporádicos de infecciones neurológicas graves. El principal reservorio de estos virus se desconoce y esto es una limitación importante a nivel epidemiológico y de control. Además, las implicaciones de la coinfección por flebovirus y *L. infantum* está poco estudiada y, hasta ahora, el único vínculo epidemiológico entre ellos es el vector común.

Hepatozoon spp. y *Babesia* spp. comprenden varias especies, muchas de las cuales son específicas de hospedador. Infectan leucocitos y eritrocitos, respectivamente, y aunque son comunes las infecciones subclínicas, pueden causar enfermedad grave y alta mortalidad. Se han descrito coinfecciones de los parásitos *Hepatozoon* spp. y *Babesia* spp. con *L. infantum* en animales domésticos y silvestres, pero todavía se desconoce el sinergismo patogénico entre ellos.

La contribución de esta tesis doctoral al conocimiento de la epidemiología de estos patógenos es presentada en cuatro capítulos. En el **Capítulo 1**, se examinó clínicamente a

gatos callejeros y perros abandonados de la Región de Murcia, y se analizaron para la infección por *L. infantum* en muestras de sangre, piel y tejido linfoide, y para la infección por *Babesia* spp. y *Hepatozoon* spp. en muestras de sangre. Usamos la Reacción en Cadena de la Polimerasa (PCR) a tiempo real, con sonda TaqMan, amplificando el ADN del kinetoplasto (kDNA) para detectar la infección por *L. infantum.* Además, se utilizó una PCR convencional de punto final amplificando secuencias codificadoras de la subunidad 18S del ARN ribosomal con cebadores genéricos, como primer paso de cribado para detectar ADN de los apicomplejos, y las muestras positivas fueron sometidas a un protocolo de PCR específica de género. Se secuenciaron los amplicones de *Babesia* spp. y *Hepatozoon* spp. y se construyeron árboles filogenéticos. Se obtuvo una estimación semicuantitativa del ADN de *L. infantum* considerando los ciclos umbrales (CTs) de la PCR a tiempo real, que están inversamente relacionados con la cantidad de secuencias de ADN diana. Se analizó el grado de concordancia entre los resultados de *L. infantum* en distintas muestras biológicas mediante el cálculo del coeficiente *kappa.*

Se detectaron signos clínicos en el 53% (112/212) de los gatos y en el 2% (2/82) de los perros. Las prevalencias de *L. infantum* en perros y gatos fueron 44% (36/82) y 21% (44/212), respectivamente. *Hepatozoon felis* se detectó en el 25% (31/123) de los gatos, *Hepatozoon canis* en el 13% (11/82) de los perros. Solo un perro estaba infectado con *Babesia* spp., identificada como *Babesia vogeli*. El análisis de secuencias de *Hepatozoon* spp. reveló tres aislados diferentes de *H. canis* y tres aislados de *H. felis*. Se detectaron infecciones mixtas de *L. infantum* con *H. canis* o *H. felis* en el 7% de los perros y 5% de los gatos, respectivamente, pero no hubo una asociación estadísticamente significativa entre las infecciones por los parásitos flagelados y esporozoarios. La prevalencia de *L. infantum* difirió en función del tipo de muestra, estación de muestreo y estado clínico de los gatos. Fue mayor en tejido linfoide y piel comparado con sangre tanto en perros como en gatos, excepto en los gatos sintomáticos, en los que fue similar. Además, la prevalencia en tejido linfoide fue mayor y los CTs de la PCR menores (indicando mayor carga parasitaria) en perros comparado con gatos, posiblemente sugiriendo un menor riesgo de desarrollar leishmaniosis sistémica en los gatos que en los perros, y con lesiones predominantemente cutáneas. Los signos clínicos compatibles con leishmaniosis felina más frecuentes en este estudio fueron lesiones cutáneas y linfadenomegalia. Sin embargo, los signos clínicos no estuvieron asociados a la infección por *L. infantum.* Además, la prevalencia de la infección por *L. infantum* en perros y gatos fue mayor en invierno, lo cual

es compatible con el largo periodo de incubación de *L. infantum* tras la típica estación de transmisión de Mayo a Octubre en la Región de Murcia. Los resultados de este estudio son importantes desde una perspectiva clínica y de diagnóstico, y apoyan el relevante papel de los gatos en la epidemiología de *L. infantum.*

El **Capítulo 2** se basa en el anterior estudio, investigando la prevalencia de *L. infantum, Hepatozoon* spp. y *Babesia* spp. en carnívoros silvestres, incluyendo zorros, tejones, garduñas, erizos, gatos monteses, meloncillos, nutrias, ginetas y mapaches. Los animales provenían de regiones del sur de España, incluyendo Murcia, Comunidad Valencia, Andalucía y Extremadura. Se extrajo el ADN de muestras de bazo, y se amplificó ADN de *L. infantum, Hepatozoon* spp. y *Babesia* spp. con los mismos protocolos de PCR utilizados en perros y gatos, descritos en el Capítulo 1. Las prevalencias globales de *L. infantum, Babesia* spp. y *Hepatozoon* spp. en fauna silvestre fueron 23%, 48% y 68%, respectivamente. Además, la PCR convencional genérica permitió la amplificación inesperada de *Cytauxzoon* spp. en un gato montés y de *Sarcocystis* spp. en una gineta. El análisis de secuencias reveló tres aislados diferentes de *Babesia vulpes* en zorros, tres aislados de *Babesia* sp. tejón tipo A en tejones, 15 aislados de *H. canis* en zorros y garduñas, tres aislados de *H. felis* en gatos monteses, y seis aislados de *H. martis* en garduñas, tejones y gatos monteses. Algunos de los aislados de *H. canis* y *H. felis* fueron idénticos a aquellos encontrados en animales domésticos de la misma región (Capítulo 1), sugiriendo un ciclo de transmisión común para estos parásitos, como también se sugiere para *L. infantum* en el estudio presentado en el Capítulo 4. La prevalencia de infección por *L. infantum* varió en función del área de estudio, el año y la especie de hospedador. Fue significativamente más prevalente en el sudeste de España (Murcia y Comunidad Valenciana) comparado con el sur y sudoeste (Andalucía y Extremadura) en zorros y tejones, y en 2013-2015 comparado con 2016-2018 en zorros. Al contrario que en perros y gatos (Capítulo 1), la infección por *L. infantum* estuvo significativa y positivamente asociada a las infecciones por *Hepatozoon* spp. y *Babesia* spp., sugiriendo que estar infectados con los apicomplejos es un factor de riesgo para infectarse con *L. infantum,* y viceversa. Son necesarios más estudios para evaluar esta relación en otras poblaciones y para clarificar los posibles mecanismos clinicopatológicos asociados a estas coinfecciones. Además, los hallazgos de este estudio contribuyen a un mejor entendimiento de la etiología y ciclos de transmisión de *Hepatozoon* spp. y *Babesia* spp.

El **Capítulo 3** se centra exclusivamente en humanos donantes de sangre y el objetivo fue estimar la prevalencia de *L. infantum* y de los flebovirus TOSV y SFSV transmitidos por vectores, evaluar la exposición a las picaduras del flebotomo *P. perniciosus* e investigar la relación entre estas infecciones y con la exposición al vector. Esto último fue realizado analizando la respuesta de anticuerpos de los donantes frente al homogenado de glándulas salivares (SGH) de *P. perniciosus* y frente a la proteína salivar recombinante rSP03B. Esta ha sido la primera vez que se analiza la respuesta humoral a esta especie vectora en humanos. Recogimos 670 muestras de sangre de 575 hemodonantes de 13 pueblos rurales y periurbanos en la Región de Murcia durante dos campañas de hemodonación en Enero-Marzo de 2017 y Septiembre-Noviembre de 2018. Se utilizó el Inmunoensayo Ligado a Enzimas (ELISA) para analizar anticuerpos frente a los antígenos salivares de *P. perniciosus* con dos protocolos de ELISA caseros y, para *L. infantum,* utilizando un test disponible comercialmente. El ADN de *Leishmania* se analizó con la PCR a tiempo real anteriormente descrita amplificando el kDNA. Se utilizaron pruebas de neutralización del virus para analizar anticuerpos frente a TOSV y SFSV. Se detectaron anticuerpos frente al SGH y/o rSP03B en el 98% de los donantes, pero la correlación entre las densidades ópticas de anticuerpos para estas preparaciones de antígeno fue solo moderada ($\rho = 0.39$). Por tanto, parece que la proteína recombinante rSP03B no es un marcador adecuado para evaluar la exposición a *P. perniciosus.* La seroprevalencia de TOSV fue del 26%, mientras que ninguno de los donantes fue seropositivo a SFSV. Las prevalencias de ELISA y PCR para *L. infantum* fueron del 1% y 2%, respectivamente, y la prevalencia global de *L. infantum* teniendo en cuenta resultados de ambas técnicas fue del 3%. Solamente un 1% de los donantes fue positivo tanto a TOSV como a *L. infantum.* Los anticuerpos frente al homogenado de glándulas salivares fue significativamente mayor en donantes positivos a TOSV y *L. infantum*. Los modelos de regresión multivariable indicaron que el riesgo de infección por TOSV fue mayor en personas mayores viviendo en zonas rurales y muestreadas en 2017. Por el contrario, la prevalencia de *L. infantum* no se asoció a ninguna variable demográfica, excepto que fue también mayor en 2017 comparada con 2018. De forma similar, los anticuerpos salivares fueron también significativamente más altos en 2017. Los seropositivos a TOSV estuvieron presentes en todos los centros de hemodonación con prevalencia variable, oscilando entre el 10% y 44%, mientras que los positivos a *L. infantum* se detectaron en seis de los trece centros de hemodonación y las prevalencias variaron entre el 3% y 10%. Estos resultados evidencian importantes diferencias en el riesgo de infección por TOSV y *L. infantum* entre áreas y años en la Región

de Murcia, lo que sugiere que las transmisiones de estos patógenos no son dependientes entre sí, y que están sujetas a marcadas diferencias interanuales. Se requieren más estudios para investigar los factores medioambientales y antrópicos asociados a la distribución de TOSV y *L. infantum.*

En el **Capítulo 4** analizamos la diversidad genética entre cepas de *L. infantum* procedentes de perros, fauna silvestre y humanos. Éstas últimas incluían muestras de casos de leishmaniosis humana visceral (VL) y cutánea (CL) y de donantes de sangre asintomáticos. Las muestras de animales provenían de animales silvestres infectados y asintomáticos, y de perros con LCan tras cultivo in vitro de los aislados de *L. infantum.* Se utilizaron dos técnicas de tipificación, la PCR seguida de secuenciación y detección de polimorfismos de nucleótido simple (SNPs), en secuencias de kDNA y espaciador transcrito interno ribosomal (ITS)-2; y la kDNA-PCR seguida de la técnica de Polimorfismos en la Longitud de los Fragmentos de Restricción (RFLP) con un panel de nueve endonucleasas. El análisis de las secuencias de ITS-2 reveló la presencia de *L. infantum* en todas las muestras excepto en un paciente latinoamericano positivo a *L. panamensis.* Se identificaron dos cepas de *L. infantum* positivas a ITS-2 que divergían en un único nucleótido. Por el contrario, el análisis de las secuencias de kDNA permitió la clasificación de *L. infantum* en 11 "genotipos-SNP" diferentes, presentando de 1 a 14 SNPs. Algunos genotipos-SNP fueron específicos de humanos o perros mientras que otros fueron compartidos entre perros, humanos y fauna silvestre. Además, un perro estaba coinfectado con dos genotipos-SNP distintos, lo que posiblemente indica coinfección con dos cepas distintas de *L. infantum.* La diversidad genética analizada mediante PCR-RFLP fue comparativamente menor y reveló únicamente cuatro genotipos diferentes (B, F, S, T). el genotipo B fue el más frecuente y los genotipos S y T no se habían reportado previamente. Los análisis filogenéticos agruparon los genotipos-SNP en tres grupos, dos de ellos conformados únicamente por cepas procedentes de humanos, y el otro agrupando cepas procedentes de humanos y animales domésticos y silvestres. No se encontró asociación entre genotipos específicos y la presentación clínica de la leishmaniosis en humanos. La diversidad genética fue mayor en los individuos sintomáticos que en los asintomáticos, y en humanos comparado con animales. Estos resultados confirman el kDNA como un marcador adecuado para investigar el polimorfismo genético, y que hay una gran heterogeneidad genética en las poblaciones de *L. infantum* del sudeste de España, incluyendo cepas específicas de hospedador y cepas comunes, lo que sugiere la existencia

de ciclos de transmisión independientes y compartidos entre animales y humanos, y los ambientes doméstico y silvestre.

En conjunto, el trabajo llevado a cabo para esta tesis doctoral contribuye al entendimiento de diversos aspectos de la epidemiologia de patógenos transmitidos por flebotomos o garrapatas en humanos y animales domésticos y silvestres del sur de España y otras áreas donde estos patógenos estén presentes. Enfatizamos la importancia de una visión global de las infecciones transmitidas por vectores, llevando el enfoque de "Una sola salud" a la investigación de la epidemiología de *L. infantum,* enfocándose en humanos y animales, y analizando la exposición al principal vector de *L. infantum* en el Oeste de Europa. Reportamos una alta prevalencia de infección por *L. infantum, Hepatozoon* spp. y *Babesia* spp. y TOSV y proporcionamos evidencia de ciclos de transmisión compartidos entre humanos, animales domésticos y animales silvestres. Esperamos que la información generada contribuya en el diseño de programas de control basados en la evidencia, para reducir el impacto de estos patógenos en las población humana y animal.

GENERAL INTRODUCTION

INTRODUCTION

Vector borne infections represent a major challenge for human and animal health worldwide, and their complexity derives from the interaction between host, vector, pathogen and environment (Figure 1). Phlebotomine sand flies are hematophagous arthropods (Diptera: Psychodidae: Phlebotominae) responsible for the transmission of the protozoan parasite *Leishmania* spp. and phleboviruses (Phenuiviridae) in the warm latitudes of the planet, including countries in the Mediterranean basin. Similarly, ticks are major vectors of human and animal diseases and they include an ample number of species adapted to widely different environmental conditions, resulting in ticks being present in almost every latitude of the planet (Brites-Neto et al., 2015; Dantas-Torres, 2008). *Babesia* spp. and *Hepatozoon* spp. are important tick-borne protozoan parasites and in Mediterranean countries they are commonly found among *Leishmania infantum* infected animals (Attipa et al., 2017; Miró et al., 2015; Tabar et al., 2008; Vilhena et al., 2013).

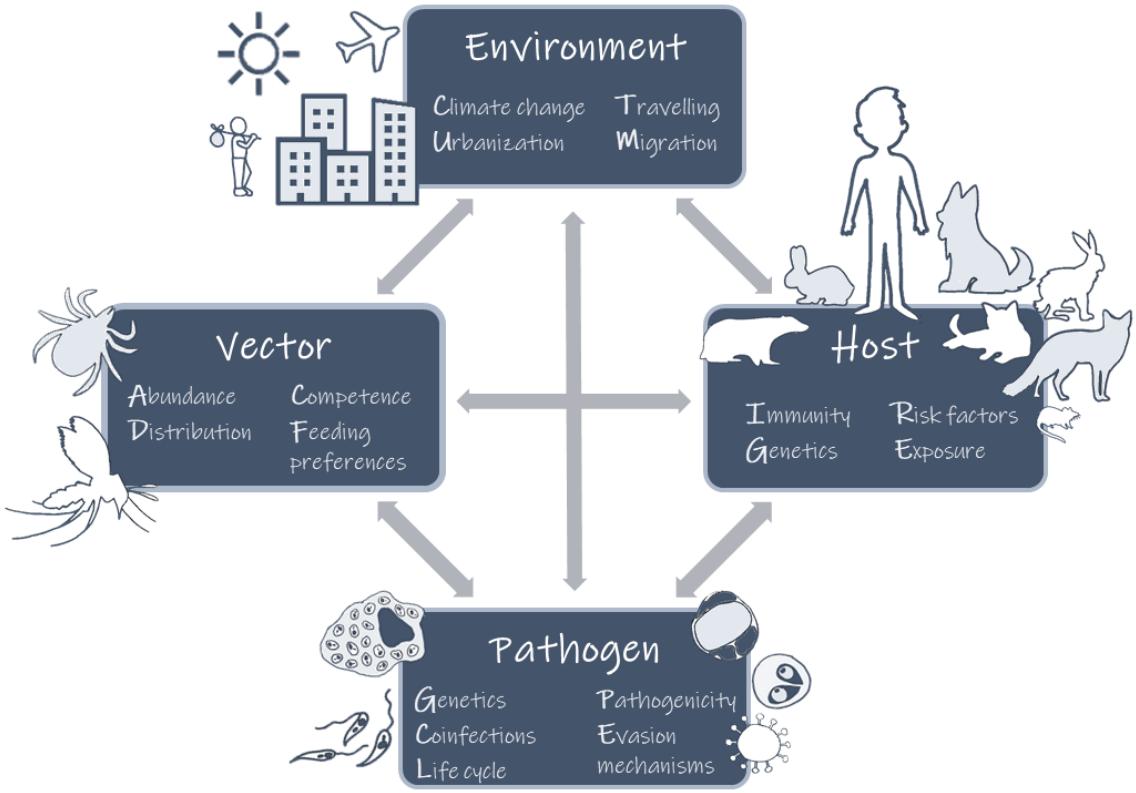

Figure 1. Diagram of vector-borne infections epidemiology involving host, vector, pathogen and environment factors.

Leishmania spp. infect reticuloendothelial cells of humans and animals and phleboviruses include neurotropic pathogens and humans are the main target host species. Among the more than 20 *Leishmania* species described worldwide, *L. infantum* is the only endemic species in Spain and causes human leishmaniosis (HumL) and canine leishmaniosis (CanL) (Martín-Sánchez et al., 2004; Fernández-Arévalo et al., 2020). Canine leishmaniosis typically presents a wide spectrum of clinical signs ranging from mild cutaneous lesions to multiorgan affection with a high mortality. In humans, leishmaniosis by *L. infantum* presents well defined clinical forms including cutaneous, visceral, mucocutaneous and mucosal leishmaniosis (CL, VL, MCL and ML, respectively), depending on the *L. infantum* strain and the host´s immune response (Colmenares et al., 2002; Gradoni et al., 2017). Dogs are the primary domestic reservoir of the parasite and the most susceptible species of infection, and humans are considered an accidental host. In Spain, HumL was traditionally described in children, associated to malnutrition and poor sanitary conditions and incidence was high until the decade of 1960s (Botet & Portús, 1993). The human immunodeficiency virus (HIV) epidemic in the 1980s saw a dramatic rise in the incidence of HumL, highlighting the tight relationship between disease development and the host's immunological status. Indeed, leishmaniosis control relies on a strong T lymphocyte mediated cellular response and most people and a large proportion of dogs may become latently infected and will not develop clinical leishmaniosis. In addition to HIV infection, HumL is an important disease in humans under immunosuppressive treatments such as organ transplant recipients and those suffering autoimmune conditions. However, HumL may also affect immunocompetent people lacking acquired immunity and exposed to a large infection challenge. This was recently exemplified in a HumL community outbreak in newly constructed residential areas in Fuenlabrada municipality in Madrid (Carrillo et al., 2013). In this unusual outbreak, lagomorphs were identified as the main reservoirs of *L. infantum* providing further evidence of the parasite's ability to infect a wide range of animals (Molina et al., 2012; Arce et al., 2013; Jiménez et al., 2014). Indeed, infection has been reported in domestic and wild host species, albeit it is typically asymptomatic (Cardoso et al., 2021; del Río et al., 2014; Millán et al., 2011). Still, their ability to transmit infection to sandflies is not known for most host species (Millán et al., 2014; Risueño et al., 2018; Alcover et al., 2020). Also, the extent to which domestic and sylvatic transmission cycles interact is a matter of debate with important disease control implications (Hong et al., 2020; Millán et al., 2014; Quinnell & Courtenay, 2009; Tomassone et al., 2018).

An aspect of interest for the scientific community is the potential relationship between *L. infantum* infections and other common pathogens but few such associations have been confirmed in animals. Our understanding of phleboviral infections is scarce compared to *L. infantum* infections. Species described in humans in Spain include Toscana virus (TOSV), Sandfly Fever Sicilian virus (SFSV), Sandfly Fever Naples virus (SFNV) and Granada virus (GRV). Infections maybe asymptomatic or associated with a flu-like syndrome except TOSV which may cause meningitis and meningoencephalitis (De Ory et al., 2009; Depaquit et al., 2010; Echevarría et al., 2003). In Spain, GRV was implicated in a mild syndrome that included fever, exanthema and acute respiratory infection (Navarro-Marí et al., 2013). Humans are considered dead-end hosts and no reservoir of infection has yet been identified (Muñoz et al., 2020). Moreover, risk factors predisposing infection are largely unknow and in Spain the distribution of infection has not been characterised in most parts of the country. Concerning *Hepatozoon* spp. and *Babesia* spp., they comprise a large number of species with a variable degree of host-specificity and pathogenicity (Giannelli et al., 2017; Matijatko et al., 2012; Penzhorn & Oosthuizen, 2020). They target leukocytes and erythrocytes, respectively, and whilst infections are often asymptomatic, they may also be life-threatening (Marchetti et al., 2009; Matijatko et al., 2012), and disease might be exacerbated in animals presenting immunosuppression or concomitant and potentially synergic infections (Baneth, 2011). Coinfection between these apicomplexan parasites and other vector-borne pathogens including *L. infantum* are common in areas where both sandflies and ticks are abundant (Attipa et al., 2017; Miró et al., 2015; Morgado et al., 2016; Otranto et al., 2009). However, the pathogenic pathways associated with coinfections between these pathogens remain unclarified.

The investigations that led to the present thesis aimed at advancing our knowledge of different aspects of the epidemiology, aetiology, diagnostic and clinical features of sand fly-borne leishmaniosis and phleboviral infections, and tick-borne *Hepatozoon* spp. and *Babesia* spp. and the relationship between these pathogens, in animals and humans from different ecosystems in southern Spain.

BIBLIOGRAPHIC REVIEW

Phlebotomine sandflies

Sandfly species and vectorial competence for *Leishmania* and phleboviruses

Among the more than 800 sand fly species recorded worldwide, more than 70 are able to transmit *Leishmania* parasites (Maroli et al., 2013; Akhoundi et al., 2016). Old World sand flies are classified in the genuses *Phlebotomus* and *Sergentomyia*. Whilst *Leishmania* spp. is transmitted only by *Phlebotomus* spp., both genuses have been incriminated in *Phlebovirus* transmission and to date phleboviruses have been detected in at least eight sand fly species in Mediterranean countries (Ayhan & Charrel, 2017). Based on their ability to transmit *Leishmania* spp., *Phlebotomus* spp. vectors were classified as "permissive" or "specific" with the former allowing development of several species and the latter restricting development of only one species (Volf & Myskova, 2007). Most of the sixteen confirmed or suspected *Leishmania* spp. vectors in Mediterranean countries (Table 1) including *Phlebotomus perniciosus* and *P. ariasi*, vectors of *Leishmania infantum* in Spain, are permissive vectors. Instead, *P. papatasi* and *P. sergenti* are specific vectors for *L. major* and *L. tropica*, agents of CL in Northern Africa and the Middle East. Most sand fly species studied so far appear to be permissive for phleboviruses including *P. ariasi, P. perniciosus, P. papatasi, P. sergenti* and *Sergentomyia minuta* present in Spain and, in Europe, the primary sandfly *Phlebovirus* vectors are *P. perniciosus*, *P. perfiliewi* and *P. papatasi* (Alkan et al., 2013).

Table 1. *Phlebotomus* spp. confirmed and suspected as *Leishmania* spp. vectors in European countries (based on ECDC sandfly fact sheet, 2020).

***Phlebotomus* species**	**Country**
P. alexandri	Spain, Azerbaijan, Armenia, Cyprus, Ukraine, Bulgaria, Romania, Greece, North Macedonia, Serbia
P. ariasi	Spain, France, Portugal, Italy
P. balcanicus	Azerbaijan, Armenia, Georgia, Ukraine, Bosnia & Herzegovina[1], Romania, Greece, North Macedonia, Montenegro, Serbia
P. halepensis	Azerbaijan, Georgia
P. kandelakii s.l.	Azerbaijan, Armenia, Georgia, Russia, Bulgaria, Greece, Montenegro
P. langeroni	Spain
P. longicuspis	Spain
P. major s.l.	Germany, Switzerland, Armenia[1], Georgia, Cyprus, Ukraine, Russia, Hungary, Bulgaria, Croatia, Italy, Malta, Bosnia & Herzegovina[1], Romania, Greece, North Macedonia, Montenegro, Serbia, Slovenia, Albania
P. mascittii	Germany, Spain, Switzerland, France, Belgium, Cyprus, Hungary, Croatia, Italy, Bosnia & Herzegovina, Slovakia, Greece, North Macedonia, Serbia, Slovenia, Austria
P. papatasi	Spain, Switzerland, France, Portugal, Azerbaijan, Armenia, Georgia, Cyprus, Ukraine, Russia, Moldova, Hungary, Bulgaria, Croatia, Italy, Malta, Bosnia & Herzegovina[1], Romania, Greece, North Macedonia, Montenegro, Serbia, Slovenia, Albania
P. perfiliewi s.l.	Armenia, Georgia, Cyprus, Ukraine, Moldova, Hungary, Bulgaria[2], Croatia, Italy, Malta, Romania, Greece, North Macedonia, Montenegro, Serbia, Albania
P. perniciosus	Germany, Spain, Switzerland, France, Portugal, Azerbaijan, Cyprus, Croatia, Italy, Malta, North Macedonia[2], Montenegro[2], Serbia, Slovenia
P. sergenti	Spain, France, Portugal, Azerbaijan, Armenia, Georgia, Cyprus, Russia, Bulgaria, Italy, Malta, Romania, Greece, North Macedonia, Serbia
P. simici	Georgia, Russia, Bulgaria, Bosnia & Herzegovina[1], Greece, North Macedonia, Montenegro, Serbia
P. similis	Azerbaijan, Ukraine, Greece, North Macedonia, Albania
P. tobbi	Azerbaijan, Armenia, Cyprus, Bulgaria, Croatia, Italy, Bosnia & Herzegovina[1], Greece, North Macedonia, Montenegro, Serbia, Albania

[1]Unpublished VectorNet (https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/vector-net) data
[2]No reference

Sandfly ecological features

Egg laying and sand fly development take place in terrestrial habitats protected from desiccation and with abundant organic matter for larvae and adults to feed on (Alexander, 2000). Adult females also require a blood meal to produce eggs and they are the only stage

able to transmit pathogens to the hosts they feed from. The whole cycle is completed in around 45 days (ECDC, 2020). Highest sand fly density is found in rural and periurban areas, where suitable microhabitats include animal burrows and shelters, caves abandoned buildings, among others (Feliciangeli et al., 2004). In warm latitudes, they can be active almost all year round, but activity in Mediterranean countries is mostly from May to October and sand fly populations typically display one or two abundance peaks depending on the area and climate (Alten et al., 2016). Sand fly species differ to some extent in their ideal ecological requirements. In Spain, *P. perniciosus* is the most abundant and widely distributed, usually in warm and dry areas. In contrast, *P. ariasi* is more adapted to cold and humid regions (Aransay et al., 2004). Sandflies are frail insects and not great flyers, and they are being mainly active between sunset and sunrise.

Recent entomological studies have been performed in Murcia Region, showing that at least five sand fly species are present and *P. perniciosus* is main vector (Gálvez et al., 2020; Muñoz et al., 2018; Risueño et al., 2017). Its distribution in spatially and temporarily variable depending on macro and microenvironmental factors, including altitude, relative humidity, ambient temperature and wind speed in the first case, and proximity and features of animal' shelter in the second case (Barón et al., 2012; Risueño et al., 2017). Studies have also shown that they feed on a wide variety of domestic and wild animals (Muñoz et al., 2019), and display some degree of feeding preferences (Pérez-Cutillas et al., 2020).

Leishmaniosis

The parasite: *Leishmania* spp.

Leishmania protozoa genus (Trypanosomatida: Trypanosomatidae) include five subgenera and up to 54 different species infecting different host species, among which 21 are pathogenic to humans (Akhoundi et al., 2016; 2017). *Leishmania* species are globally distributed mostly between latitudes 45ºN and 55ºS (WHO, 2019) and have been detected in the five continents, although some subgenera, i.e., *Sauroleishmania* and *Viannia*, are restricted to the Old and New World, respectively. Moreover, *L. infantum* (syn. *L. chagasi*) was introduced in the Americas from European colonizers (Kuhls et al., 2011; Leblois et al., 2011; Boité et al., 2019).

They are heteroxenous parasites transmitted by hematophagous sand flies, presenting a biphasic life cycle with two distinct parasitic forms: the promastigote, which is the infective stage in the vector and the amastigote stage present in the host´s reticuloendothelial cells (Figure 2). Infected cells are disseminated by the vascular and lymphatic systems colonising every tissue and organs, and they can be readily detected in blood, skin, lymphoid tissues including spleen, lymph nodes and bone marrow, liver, urine, semen, saliva, conjunctiva, hair, among others (Caldas et al., 2020; Ferreira et al., 2013; Fisa et al., 2008; Riera & Valladares, 1996; Sabzevari et al., 2017; Strauss-Ayali et al., 2004). The risk of developing disease depends on the host immune response, and the incubation period in susceptible hosts is around 2 to 3 months (Oliva et al., 2006; Piscopo & Azopardi, 2007).

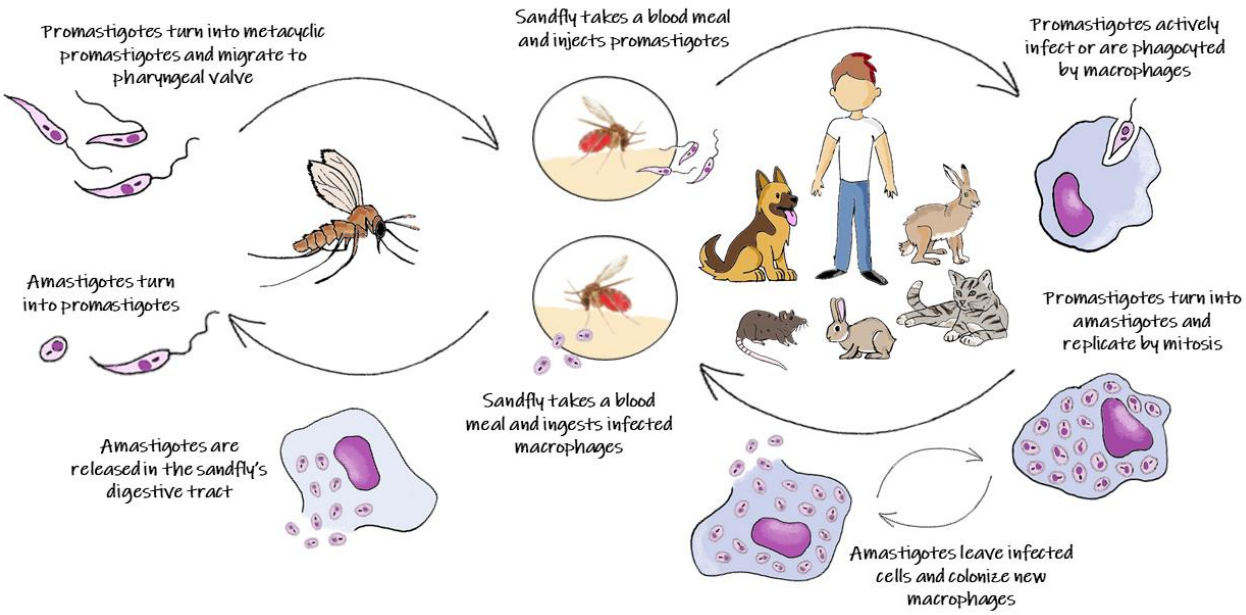

Figure 2. Life cycle of *Leishmania* spp.

Human leishmaniosis

Human leishmaniosis (HumL) is a neglected tropical disease (Alvar et al., 2006), endemic in more than 98 countries and disproportionately affecting the poorest regions, mostly from African, Asian and American continents. Moreover, climate change, globalization and conflict are causing the spread of *Leishmania* spp. into previously non-endemic territories (Jacobson, 2011; Söbirk et al., 2018; Wall et al., 2012).

Human leishmaniosis may develop as four clinical forms: visceral (VL), cutaneous (CL), mucocutaneous (MCL) and mucosal (ML) leishmaniosis (Gradoni et al., 2017).

Cutaneous leishmaniosis is the most frequent condition consisting of skin lesions and severity ranges from a few self-healing to multiple disseminated lesions, and it is caused by multiple *Leishmania* species, such as *L. major*, *L. tropica* complex (*L. tropica* and *L. aethiopica*), *L. amazonensis* and *L. mexicana* from *L. mexicana* complex, *L. braziliensis*, *L. guyanensis* and *L. donovani* complex including *L. infantum* and *L. donovani sensu stricto* (s.s.). Disseminated cutaneous leishmaniosis is mainly caused by *L. aethiopica*, *L. amazonensis* and *L. mexicana* (Bañuls et al., 2011). Mucocutaneous leishmaniosis occurs when cutaneous lesions extend to the mucous membranes, and it is associated with species from *L. braziliensis*, *L. mexicana*, and *L. guyanensis* complexes (Guerra et al., 2011). Mucosal leishmaniosis is characterized by lesions in the oral and aerodigestive tract mucosa in the absence of previous cutaneous signs (Aliaga et al., 2019; Gradoni et al., 2017). Finally, VL (also known as "kala-azar" in the Indian subcontinent) is mainly caused by species of the *L. donovani* complex, and it is the most severe clinical form of HumL, fatal in most cases if left untreated (Alvar et al., 2012). Visceral leishmaniosis clinical signs typically include fever, asthenia, weight loss, splenomegaly, hepatomegaly and pancytopenia (Singh et al., 2014). Patients recovering from VL caused by *L. donovani* s.s. may develop "post-kala azar dermal leishmaniosis" (PKDL), consisting of skin lesions in the face that can extend to other parts of the body. As it can be recognised from these classifications, several *Leishmania* species are able to cause more than one syndrome. Moreover, atypical presentations also occur and may include gastrointestinal, genital, aerodigestive tract, or cutaneous involvement distinct to PKDL in VL cases, and zosteriform, verrucous, erysipeloid, lupoid, and other unusual CL skin lesions (Ayala et al., 2017; Diro et al., 2015; Meireles et al., 2017; Mohammed et al., 2021). The annual incidences of VL and CL are 0.3 and 1.0 million new cases, respectively (Gradoni et al., 2017), probably underestimated, since in several countries its notification is not mandatory (Alvar et al., 2012).

Human leishmanioses are classified as anthroponotic or zoonotic, depending on whether the main reservoirs hosts are humans or animals, respectively (WHO, 2010). In Europe, *L. infantum* is the only endemic species and is the causative agent of zoonotic HumL (Pratlong et al., 2013), and the dog is the main domestic reservoir of the parasite. Sporadic cases of anthroponotic *L. tropica* have also been described in Crete and the Ionian islands (Christodoulou et al., 2012; Frank et al., 1993; Stratigos et al., 1980). Moreover, clinical

cases involving other imported *Leishmania* species among tourists and migrants are not uncommon (Fernández-Arévalo et al., 2021; Khan et al., 2019).

Immunodeficiency is a major risk factor for development of clinical leishmaniosis and include co-infection with human immunodeficiency virus (HIV) (Alvar et al., 2008) and immunosuppressive chemotherapy in organ transplant recipients and autoimmune conditions (Basset et al., 2005; Fletcher et al., 2015; Michel et al., 2011; Pittalis et al., 2006). A few years ago, *Leishmania*-HIV coinfection used to constitute the primary risk factor for hospitalization but, during the last years, HIV-negative HumL hospitalizations cases have increased, mainly VL forms (Herrador et al., 2015). Moreover, the vast majority of human cases in the previously mentioned community outbreak in Madrid were immunocompetent individuals (Arce et al., 2013). Therefore, several factors mediate the symptomatic/asymptomatic status, including *Leishmania* species and strains involved (Hide et al., 2013), immunological and nutritional status of the host and genetic traits.

Asymptomatic carriers are an important study subject for a better understanding of the epidemiology of leishmaniosis (Bañuls et al., 2011). The majority of immunocompetent *L. infantum*-positive humans in endemic areas present a subclinical or cryptic infection. Several studies in blood donors from Mediterranean areas have shown that *L. infantum* is present in this collective (Riera et al., 2004; Riera et al., 2008; Chitimia et al., 2011; Pérez-Cutillas et al., 2015; Aliaga et al., 2019; Ortalli et al., 2020). Parasite load in blood from asymptomatic infected individuals is usually very low (<1 parasite/ml of blood) (Jiménez-Marco et al., 2018; Mary et al., 2004), and detection of infection requires highly sensitive molecular methods such as the real-time polymerase chain reaction (PCR) targeting highly repetitive sequences. Moreover, most infected healthy individuals do not develop a humoral serological immune response (Aliaga et al., 2019). In southern European countries, serology and PCR prevalences in asymptomatic people range from 0 to 56% and from 0 to 58%, respectively (Colomba et al., 2005; Ibarra-Meneses et al., 2019; Martín-Sánchez et al., 2004; Riera et al., 2008; Scarlata et al., 2008). There has been much debate concerning the risk of transmission of *Leishmania* parasites through blood-transfusion from asymptomatically infected donors (Jiménez-Marco et al., 2016). In Europe, blood is leukodepleted and other pathogen reduction strategies are implemented, thus reducing the risk of parasite transmission from blood transfusion (Riera et al., 2008; Jiménez-Marco et al., 2018; Kyriakou et al., 2003). Considering this, it is highly improbable that they play an important role in leishmaniosis epidemiology. Moreover, a recent study indicated no

transmission from *L. infantum* to *P. perniciosus* from asymptomatically infected, immunocompetent individuals in Spain, in contrast to those coinfected with HIV (Molina et al., 2020). Still, Ferreira et al. (2018) demonstrated *L. infantum* transmission to *Lutzomyia longipalpis* sandflies from asymptomatically infected humans in Brazil.

The distribution of human leishmaniosis in endemic areas is highly heterogeneous and it is strongly linked to the abundance of infected vectors. The risk of *L. infantum* infection in Europe is greatest in rural and periurban areas that meet the ecological conditions for sand flies to complete their life cycle (Garrote et al., 2004; Moral et al., 2002). In these areas, the risk of infection depends on exposure to infected sand flies, and increases with age and behavioural factors such as working place (indoors or outdoors) and ownership of infected dogs (Pérez-Cutillas et al., 2015). Some studies also report that positive cases are higher in men than in women, which might be explained by behavioural differences and poorly understood biological factors (Acedo-Sánchez et al., 1996; Biglino et al., 2010; Cloots et al., 2020).

Canine leishmaniosis

The dog is the main reservoir of *L. infantum* in the Mediterranean domestic context (Solano-Gallego et al., 2001). This means that they can effectively maintain the transmission cycle in the absence of other host species. Canine leishmaniosis (CanL) is endemic in southern Europe, where it is estimated that 2.5 million dogs are infected by *L. infantum* (Moreno and Alvar, 2002), and occasional infections by *L. major* and *L. tropica* have been described in Crete, the Middle East and North Africa (Baneth et al., 2017; Ntais et al., 2013). Countries from non-endemic northern Europe have reported isolated cases of CanL, the majority of them imported, but also a few autochthonous cases have been detected in Hungary, Austria, the Netherlands, Czech Republic, Finland, Germany and Slovenia, where dogs were supposed to be infected by vertical and/or venereal transmission, through wounds or by sandflies bites (Díaz-Espiñeira et al., 1997; Farkas et al., 2011; Karkamo et al., 2014; Kotnik, 2020; Naucke & Lorentz, 2012; Naucke et al., 2016; Obwaller et al., 2016; Svobodova et al., 2017; Tánczos et al., 2012). Moreover, CanL is being increasingly detected in periendemic countries such a Romania (Mihalca et al., 2019). In southern European Mediterranean countries, CanL PCR and antibody prevalences can reach 80% and more than 50%, respectively (Aoun et al., 2009; Berrahal et al., 1996; Franco et al., 2011; Gálvez et al., 2020; Solano-Gallego et al., 2001). In contrast, only a 10-

15% of infected dogs will develop clinical disease (Solano-Gallego et al., 2001; Figure 3). As mentioned before, many subclinically infected dogs behave as asymptomatic carriers of the parasite and play an important role in *L. infantum* epidemiology, as they are able to transmit the parasite to sandflies (Borja et al., 2016; Laurenti et al., 2013; Molina et al., 1994). However, infectiousness is primarily associated to symptomatic dogs (Michalsky et al., 2007; Verçosa et al., 2008) and to skin and blood parasite loads (Borja et al., 2016).

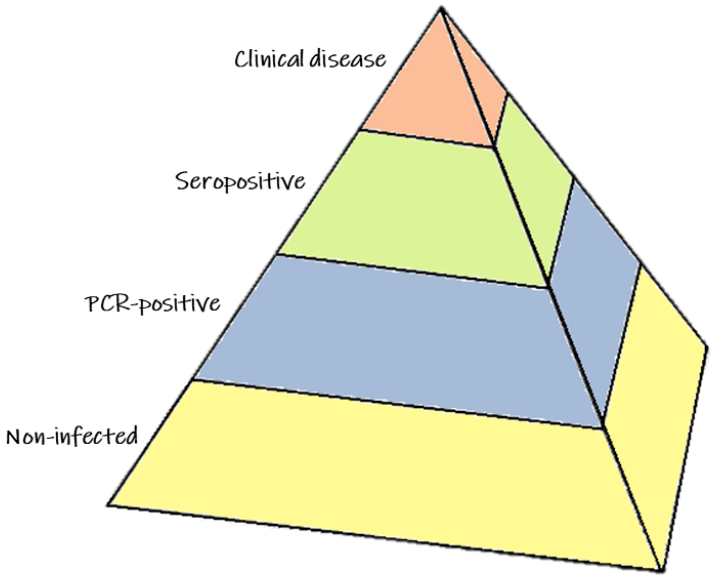

Figure 3. Typical distribution of *Leishmania* infection distribution among canine population in endemic areas. Based on Baneth et al. (2008).

Canine leishmaniosis is a multisystemic, immunologic disease that has a variety of clinical manifestations that include: general signs such as lymphadenomegaly, weight loss, lethargy, pale mucous membranes; cutaneous lesions including papular/exfoliative/ulcerative dermatitis, onychogryphosis; ocular lesions, i.e., blepharitis, conjunctivitis, uveitis; and other less frequent clinical signs including epistaxis, vascular or neurological disorders. Laboratory abnormalities can include mild to moderate non-regenerative anemia, thrombocytopenia, leukocytosis or leukopenia, hyperglobulinemia and hypoalbuminemia, increased levels of hepatic enzymes, proteinuria, and renal azotaemia (Baneth et al., 2008; Solano-Gallego et al., 2011).

Disease outcome mostly depends on the host's immune response. Thus, a cellular immune response mediated by type 1 T helper lymphocytes (Th1) is considered as protective, whereas humoral immune response mediated by type 2 T helper lymphocytes

(Th2) is linked to progression of the disease, since a high antibody production and the deposit of antigen-antibody complexes lead to appearance of clinical signs. Susceptibility or resistance to clinical disease seems to be genetically influenced (Ferrer et al., 2002). Some dog breeds are more susceptible to the disease (Miranda et al., 2008), whereas others i.e., the Ibizian hound, have proven to be more resistant (Solano-Gallego et al., 2000).

Generally, low antibody levels are associated to subclinical infections, whereas antibody levels are high in symptomatic cases (Saridomichelakis, 2009). Some seropositive dogs with low parasite burdens may progress favourably without the need of treatment. Moreover, treatment of infected dogs does not assure parasitological cure and relapses are common (Francino et al., 2006). However, treating dogs with clinical leishmaniosis is important from a welfare and zoonotic perspective and epidemiologically, as it reduces dogs infectivity to sandflies (Miró et al., 2011).

Feline leishmaniosis

Cats are considered to be more resistant to leishmaniosis than their canine counterparts, since relatively few clinical feline leishmaniosis (FeL) cases are diagnosed and mostly in immunocompromised cats (Chatzis et al., 2004; Pennisi & Persichetti, 2018; Sarkari et al., 2009). It has been postulated that immunocompetent cats present a more efficient Th1 immune response compared to dogs (Day, 2016; Maia & Campino, 2011), but there is still little information concerning the immunological mechanisms of cats against *Leishmania* spp. infection. Authors report the predominance of cutaneous vs. visceral signs in cats with FeL (Solano-Gallego et al., 2007). Thus, the most common clinical manifestations of FeL are dermatological lesions and lymphadenomegaly, followed by ocular and oral lesions and general symptoms such as lethargy and weight loss. More rarely, other signs may be present, including hepatomegaly, vomiting, diarrhoea, splenomegaly, or respiratory signs (Pennisi et al., 2015).

L. infantum is the most common *Leishmania* species in cats (Pennisi & Persichetti, 2018), although some studies report the detection of *L. tropica* in cats from Turkey and *L. major* in cats from Turkey and Portugal (Pasa et al., 2015; Pereira et al., 2020). The advent of molecular methods has shown that, in *L. infantum* endemic countries, FeL is more prevalent than previously considered, reaching values as high as 68.5% (reviewed at Pennisi et al., 2015; Cardoso et al., 2021). Generally, FeL is less prevalent than CanL (Maia et al., 2010; Otranto et al., 2017; Tabar et al., 2008, 2009), although Baneth et al. (2020)

reported similar prevalence in sheltered dogs and cats in Israel. Results might vary according to the studied area, population origin, clinical status or diagnostic protocol. The role of cats in the epidemiology of infections remains controversial. To consider a certain species as a primary reservoir, several conditions have to be met: infection in the host species must be widespread, it has to be infectious to sandflies, and it should be able to maintain parasite's circulation in the absence of the main reservoir, the dog (Quinnell & Courtenay, 2009; WHO, 2010). Some studies have demonstrated the ability of cats to infect *P. perniciosus* and *Lutzomyia longipalpis* sandflies by xenodiagnostic experiments (Maroli et al., 2007; Mendonça et al., 2020) and genetic studies have confirmed feline infection with *L. infantum* zymodeme MON-1, the most frequently found in humans and dogs (Baneth et al., 2008; Grevot et al., 2005; Ozon et al., 1998; Pochole et al., 2012), and Maia et al. (2015) reported a FeL clinical case caused by *L. infantum* genotype E, previously isolated from dogs and immunocompromised humans (Cortes et al., 2006). Considering this, together with the fact that feline populations are large, some authors argue that cats are an important reservoir of *L. infantum* infection in the domestic environment (Martín-Sánchez et al., 2007; Gramiccia & Gradoni, 2005; Solano-Gallego et al., 2007; Maia et al., 2008). However, more studies are needed to investigate this issue further, but field studies are constrained by the presence of dog reservoirs of infection, in most cat habitats.

L. infantum infection in other domestic and wildlife hosts

Apart from dogs and cats, *L. infantum* has been detected in other domestic and wild animals (Cardoso et al., 2021) although clinical signs are not frequently reported. Also, their role as reservoirs of the parasite and their contribution to the epidemiology of leishmaniosis is unknown for most species (Maia & Campino, 2011; Quinnell & Courtenay, 2009).

In Europe, *L. infantum* infection has been detected in several wild host species, including canids, felids, mustelids, lagomorphs, rodents and other mammals, and also some bird species (Table 2). However, apart from dogs and domestic cats, only black rats (*Rattus rattus*), European rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) have proven to be infectious to sandflies in experimental conditions (Molina et al., 2012; Jiménez et al., 2014; Svodobá et al., 2003). Moreover, lagomorphs were the primary reservoir of *L. infantum* infection in the previously mentioned HumL outbreak of Fuenlabrada, Madrid (Arce et al., 2013). On the other hand, the red fox was considered as

a potential reservoir of *L. infantum* zymodeme MON-1 in the study of Campino et al. (2006), and the same happens in foxes from Spain (Lledó et al., 2015). This may suggest that domestic and sylvatic transmission cycles are connected. However, no other animal species apart from the dog has fulfilled the above-mentioned criteria.

Table 2. Origin and prevalence of *Leishmania infantum*-positive wild animals in European countries.

Family	Species	Country	Prevalence[1]
Order Carnivora			
Canidae	Golden jackal (*Canis aureus*)	Serbia, Georgia, Romania	1.9-6.9%
	Gray wolf[2] (*Canis lupus*)	Croatia, Portugal, Spain, Italy	CR[3], 4.1-46.2%
	Red fox (*Vulpes vulpes*)	Italy, Spain, Portugal, France, Greece, Georgia	CR, 1.3-59.6%
Felidae	European wildcat (*Felis silvestris*)	Spain	CR
	Iberian lynx (*Lynx pardinus*)	Spain	CR
	Barbary lion[2] (*Panthera leo*)	France	CR
	Tiger[2] (*Panthera tigris*)	Italy	CR, 45%
Herpestidae	Egyptian mongoose (*Herpestes ichneumon*)	Portugal, Spain	CR, 4.7-28.6%
Mustelidae	Eurasian otter (*Lutra lutra*)	Spain	CR, 70%
	Beech marten (*Martes foina*)	Spain	CR, 13-30%
	European pine marten (*Martes martes*)	Spain	CR, 30-39.1%
	European badger (*Meles meles*)	Spain, Italy	8.0-53.3%
	European mink (*Mustela lutreola*)	Spain	CR
	Polecat (*Mustela putorius*)	Spain	CR
	Domesticated ferret[4] (*Mustela putorius furo*)	Spain	CR
	Stoat (*Mustela erminea*)	Spain	CR
	American mink[4] (*Neovison vison*)	Greece, Spain	CR, 20-21.4%
Ursidae	Brown bear[2] (*Ursus arctos*)	Spain	CR
Viverridae	Common genet (*Genetta genetta*)	Spain	CR, 9.1-40%
Procyonidae	Raccoon (*Procyon lotor*)	Spain	CR
Order Chiroptera			
Vespertilionidae	Common pipistrelle (*Pipistrellus pipistrellus*)	Spain	59.3%
Order Diprotodontia			
Macropodidae	Bennett's wallaby[2] (*Macropus rufogriseus*)	Spain	CR
Order Eulipotyphla			
Erinaceidae	European hedgehog (*Erinaceus europaeus*)	Spain	CR, 34.4%
Soricidae	Greater white-toothed shrew (*Crocidura russula*)	Spain	13.3%

Table 2 (continued). Origin and prevalence of *Leishmania infantum*-positive wild animals in European countries.

Family	Species	Country	Prevalence[1]
Order Lagomorpha			
Leporidae	European hare (*Lepus europaeus*)	Spain, Greece, Italy	0.9-64.3%
	Iberian hare (*Lepus granatensis*)	Spain	CR, 31.9-74.1%
	European rabbit[4] (*Oryctolagyus cuniculus*)	Spain, Greece, Italy	0.6-82.6%
	Brown hare (*Lepus castroviejoi*)	Spain	CR
Order Perissodactyla			
Equidae	Horse[4] (*Equus ferus caballus*)	Germany, Portugal, Greece Switzerland, Spain, Italy	CR, 0.4-14.4%
Order Primates			
Hominidae	North-West Bornean orangutan[2] (*Pongo pugmaeus pygmaeus*)	Spain	CR
Order Rodentia			
Muridae	Wood mouse (*Apodemus sylvaticus*)	Spain	18.8-20.8%
	House mouse (*Mus musculus*)	Portugal, Spain, Greece	CR, 24.2-88.9%
	Algerian mouse (*Mus spretus*)	Spain	4.3-42.9%
	Brown rat (*Rattus norvegicus*)	Cyprus, Greece, Portugal, Spain	CR, 5.5-70%
	Black rat (*Rattus rattus*)	Cyprus, Italy, Spain, Greece	CR, 11.2-25%
Sciuridae	Eurasian red squirrel (*Sciurus vulgaris*)	Spain	20%

[1] Including serological and molecular techniques.

[2] Include captive animals.

[3] CR – Case reports. Positive animals from studies analysing less than 10 animals were considered as case reports.

[4] Include domestic or farm animals.

Diagnosis of leishmaniosis

In spite of recent advances, diagnosing leishmaniosis is still challenging due to the variety of clinical signs, many of which are not specific, and the considerable proportion of subclinical infections, characterized by low antibody titres and parasite loads.

Direct techniques searching for the parasite include optical microscopy of colour/immunological stained smears obtained from tissue imprints, lymph nodes or bone marrow aspirates, and in vitro culture of tissue samples, including spleen, lymph nodes, bone marrow, liver or skin (Figure 4). Parasite DNA can be directly detected by molecular

methods like the PCR, which has a high diagnostic sensitivity and specificity (Francino et al., 2006). Quantitative PCR (qPCR) allows the estimation of the parasite load and detection limits of some assays are less than one parasite per millilitre of blood (Dantas-Torres et al., 2017).

In addition, indirect serological diagnostic techniques detect host-specific antibodies against parasite antigens (Montalvo et al., 2012). The most common methods available include indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), Western Blot (WB), direct agglutination test (DAT) and rapid immunochromatographic tests (RICT). They employ crude promastigotes antigens, soluble parasite extracts or recombinant proteins (Ryan et al., 2002; Romero et al., 2004; Chappuis et al., 2004). These techniques are clinically useful as a first diagnostic approach, since individuals with clinical leishmaniosis develop a strong humoral response, and seroconversion can be detected soon after infection (Moreno & Alvar, 2002).

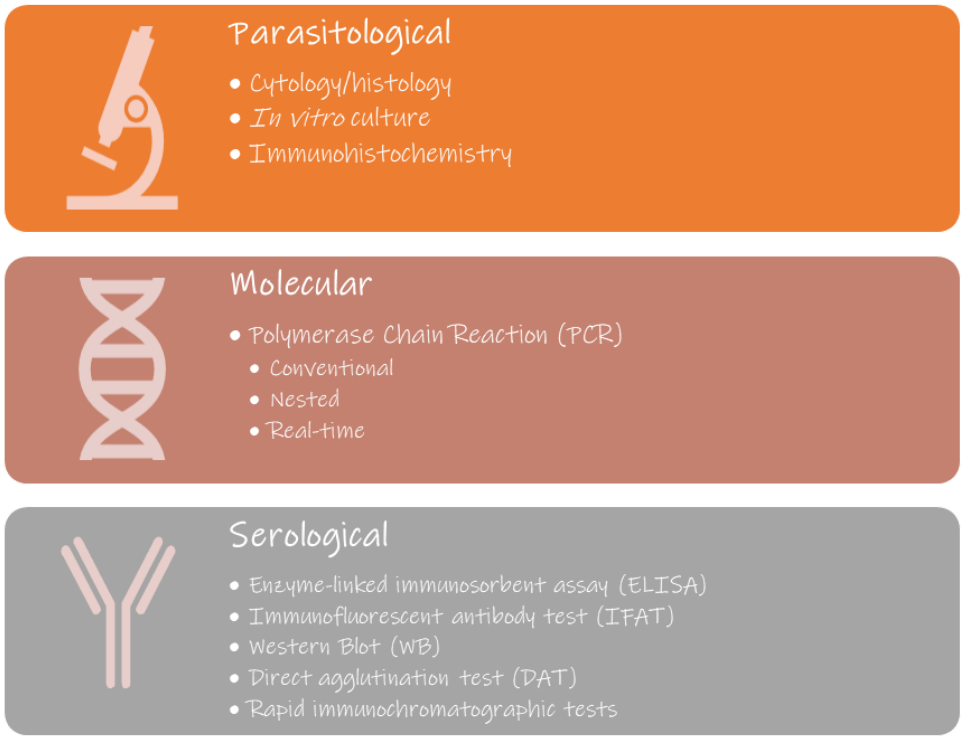

Figure 4. Leishmaniosis diagnostic methods.

Molecular techniques and markers and type of sample

The development and performance of diagnostic methods and, particularly, PCR-based molecular techniques, have changed our understanding of *Leishmania* spp. infection. They

provide greater sensitivity and specificity to detect subclinical infections, compared to parasite detection by optical microscopy (Saridomichelakis et al., 2005), and they allow species identification (Akkafa et al., 2008). As mentioned above, qPCR is the most rapid and sensitive technique for parasite DNA detection and characterization from clinical samples, treatment monitorization and vaccine trials (Cortes et al., 2004; Schönian et al., 2003).

Several DNA sequences can be targeted by PCR assays, and those present in multiple copies provide high PCR sensitivity, specially kinetoplast minicircle DNA (kDNA), which is present in around 10,000 copies per parasite (Francino et al., 2006). Other target genes from chromosomal DNA include ribosomal RNA (rRNA), internal transcribed spacer (ITS)-1 and ITS-2 (de Almeida et al., 2011; Schönian et al., 2003), mini-exon (Marfurt et al., 2003; Mauricio et al., 2004), gp63 (Mauricio et al., 2004) or heat shock protein (hsp)-70 (Montalvo et al., 2012).

As mentioned before, kDNA-PCR detection limit is less than one parasite per ml of blood (Francino et al., 2006; Dantas-Torres et al., 2016; Mary et al., 2004). It has been argued that the presence of such small DNA amounts could be due to residual *Leishmania* DNA in macrophages after its destruction and therefore not necessarily indicating active infection (Mary et al., 2004). More recent investigations say that PCR-detected DNA comes from live parasites, since nucleic acids degrade promptly after the parasite's death (de la Llave et al., 2011; Prina et al., 2007). Considering all this, a PCR threshold cycle (CT) of 38 is generally considered the limit of PCR positivity, since this CT approximates a single copy of the target DNA (Mackay et al., 2007), although some studies consider CTs up to 45 as positive (Jiménez-Marco et al., 2018).

The biological sample from which DNA for PCR analysis is used is critical for successful diagnosis of *L. infantum* infection, particularly in subclinically infected individuals. *Leishmania* infects tissue macrophages so lymphoid samples such as bone marrow, spleen and lymph node provide highest diagnostic sensitivity (Quaresma et al., 2009). However, in contrast to subclinically infected individuals, those with clinical leishmaniosis typically have parasitaemia so blood samples may offer a similar diagnostic sensitivity and are more convenient to take than lymphoid tissue samples (Francino et al., 2006; Manna et al., 2004). Other types of samples that have been used and proved useful for *Leishmania* diagnosis are conjunctival or oral swabs, reaching sensitivities over 90%

and 75% (Ferreira et al., 2013; Pilatti et al., 2009; Strauss-Ayali et al., 2004) and other biological samples from cardiorespiratory, digestive, reproductive and urinary systems (Boechat et al., 2020; Goncalvez et al., 2018; Guillén et al., 2020; Solano-Gallego et al., 2007).

Genetic variability of *Leishmania* spp. and *L. infantum*

Characterization of the *Leishmania* species involved in clinical cases is highly relevant for both clinical and epidemiological reasons (Akkafa et al., 2008; de Andrade et al., 2006; Rotureau et al., 2006; Schonian et al., 2003). This is particularly important in areas where different species of *Leishmania* coexist and are transmitted sympatrically and thus, diagnosis based on geographical location is not feasible (Cupolillo et al., 2003; Rocha et al., 2016). Furthermore, genetic variants can be found for a particular *Leishmania* species, and studies performed so far show a wide genetic heterogeneity for *L. infantum*. In the study of Oryan et al. (2013), the distinct *L. major* isolates from CL patients clustered together according to geographical origin and type of clinical manifestation, and Cortes et al. (2006) found that *L. infantum* genotypes A and B were correlated with immunocompromised and immunocompetent patients, respectively. However, in the study of da Silva et al. (2015) in Brazil, no relationship was found between the genetic variants of *L. infantum* and demographic or clinical variables. Mixed infections with different strains have also been reported in humans and dogs (Ortuño et al., 2019; Fernández-Arévalo et al., 2021).

Genetic exchange is one of the sources of intra-specific variability. Sexual reproduction of *Leishmania* has not been experimentally proved yet, although promastigote fusion and double nuclear DNA in amastigotes have been reported (Kreutzer et al., 1994; Lanotte & Rioux, 1990; Youssef et al., 1997). Since gene flow does not occur with the same intensity in all geographical areas, the degree of *Leishmania* polymorphism varies between countries (Martín-Sánchez et al., 2004; Cortes et al., 2006).

Typing methods, molecular markers and *L. infantum* genetic variants

There are several techniques to identify genetic variability in *Leishmania* species, presenting different discriminatory power. At present, the best standardized method to differentiate species and subspecies is isoenzyme analysis (Multilocus Enzyme

Electrophoresis, MLEE), based on the analysis of the isoenzymatic profile of *Leishmania* spp. strains (Rioux et al., 1990), and it is considered by some authors as the "gold standard" for *Leishmania* species identification (Schönian et al., 2003; Van der Auwera & Dujardin, 2015). It was introduced in 1980 and it has allowed the classification of *L. infantum* variants from different hosts and vector species into zymodemes, according to the Montpellier nomenclature (Campino et al., 2006). So far, more than 30 *L. infantum* zymodemes have been described, among which 21 have never been isolated from vectors. Zymodeme MON-1 is the most frequent and widely distributed, present in more than 30 countries of the New and Old World. It represents 70% of all human VL strains and 50% of those from human HIV-positive patients (Chicharro et al., 2003; Gallego et al., 2001; Morales et al., 2001). In the Mediterranean basin, MON-1 zymodeme is also the most common of at least the 12 described. It is also the most frequent zymodeme in dogs (Martín-Sánchez et al., 2004; Pratlong et al., 2004), although in sandflies is present in lower proportions (Martín-Sánchez et al., 1994).

L. infantum zymodeme variability in Spain is high (Chicharro et al., 2003; Cortes et al., 2011), and it could be due to sexual recombination (Martín-Sánchez et al., 2004). In the study of Martín-Sánchez et al. (2004) in southern Spain, they found up to 20 different zymodemes in isolates from humans, dogs, black rats and sandflies. The latter were holding the greatest *L. infantum* zymodemes diversity (16 zymodemes), compared to humans (10 zymodemes) and dogs (4 zymodemes). Even though the majority belonged to MON-1 zymodeme, the high degree of polymorphism contrasts with other studies in Portugal (Campino et al., 2006), and it is similar to one in Italy, with 11 different zymodemes being identified (Gramiccia, 1995). Pratlong et al. (2004) confirms MON-1 as the most frequent etiological agent in dogs, humans, foxes and sandflies. Zymodemes diversity seems to be higher in VL/HIV coinfected patients (Gallego et al., 2001; Pratlong et al., 2004).

Although MLEE is still the reference typing technique for *Leishmania* spp. and has greatly contributed to our knowledge of leishmaniosis epidemiology (Martín-Sánchez et al., 2004), it is a laborious and expensive method only available at few laboratories with *Leishmania* culturing facilities. Moreover, its discriminatory scope is limited compared to current methods based on DNA sequences analysis (Hide et al., 2001) which are now widely available, cheaper and can be used on samples without previous culturing (van der Auwera & Dujardin, 2015). The most commonly used molecular typing methods are PCR amplification of specific genes combined with restriction fragment length polymorphism

analysis (PCR-RFLP) (Cortes et al., 2006; El Hamouchi et al., 2017; Silva et al., 2010) or sequencing (PCR-sequencing). Other techniques include PCR-single strand conformation polymorphism (PCR-SSCP) analysis (El Tai et al., 2000) or random amplification of polymorphic DNA (RAPD) (Toledo et al., 2002).

In PCR-RFLP, the PCR amplicon is digested by one or more restriction enzymes, followed by the analysis of the fragments' size. Species identification by PCR-RFLP is less expensive and time-consuming than isoenzyme analysis and it has enough sensitivity to directly detect parasites from clinical samples, as well as to identify almost all the clinically relevant species (Schönian et al., 2003). Therefore, it is an efficient method for species identification (de Andrade et al., 2006; Ferroglio et al., 2006; Rotureau et al., 2006). The more restriction enzymes used, the greater molecular diversity is revealed, and variants are found within the same *Leishmania* zymodeme (Cortes et al., 2006). Rotureau et al. (2006) found that, among all restriction enzymes, *Rsa*I was the most efficient for distinguishing species, except for *L. (V.) guyanensis* and *L. (V.) lainsoni*. In contrast to results of Schönian et al. (2003), restriction with *Hae*III enzyme was not enough to clearly distinguish all *Viannia* species. According to Volpini et al. (2004), kDNA PCR-RFLP allows the distinction between *L. amazonensis, L. braziliensis* and *L. infantum* (syn. *L chagasi*).

Multilocus sequence typing (MLST) is also used to study *Leishmania* spp. diversity. It is based on sequencing and analysis of several single-copy genes, but its low sensitivity impedes its use on clinical samples (van der Auwera & Dujardin, 2015). Similarly, multilocus microsatellite analysis (MLMA) differentiates species according to microsatellite sequence size polymorphisms. Using this technique, Kuhls et al. (2008) identified three geographical groups within the MON-1 human zymodeme. In Europe, Montoya et al. (2007) detected different genotypes in sandflies and dogs from Spain and Rougeron et al. (2011) identified *L. infantum* variants in isolates from humans and dogs in Sudan. In addition, MLST reproducibility is high and, since DNA sequencing is now more easily available, some authors consider that it will replace MLEE as reference method for *Leishmania* species identification (Lauthier et al., 2020). Recent investigations confirm MLST capacity to distinguish between isolates from the same species (El Baidouri et al., 2013) and the same technique was employed to differentiate strains in a clinical outbreak due to *L. (Viannia) braziliensis* in Brazil (Marlow et al., 2014).

Finally, matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) is lately been employed for species determination. It also requires prior culturing of *Leishmania* from clinical or sand fly samples and it lacked precision for controversial *Leishmania/Viannia* complexes and it does not recognize intra-specific variability (refs in Fernandez-Arevalo 2021; Fernández-Arévalo et al., 2021).

Several molecular markers have been used for studying *Leishmania* species and strain genetic divergence, which differ on the degree of DNA variability. Among those employed are nuclear DNA genes such as ribosomal small subunit (SSU)-rRNA, ITS1, ITS2, mini-exon, gp63, heat-shock proteins and microsatellite DNA as well as extrachromosomal DNA like kDNA minicircles (Akhoundi et al., 2017). The latter has been extensively used because in addition to its high copy number it displays a high sequence variability (Marfurt et al., 2003). The kDNA hipervariable region permits the identification of intraspecific genetic variants (Chicharro et al., 2002; Cortes et al., 2006; Ferroglio et al., 2006) (Figure 5), albeit it impedes the design of universal primers for all *Leishmania* species (Marfurt et al., 2003). Cortes et al. (2006) employed kDNA-PCR-RFLP in isolates from humans, dogs and sandflies from Portugal, and in strains from Spain, France, Malta, Brazil, Sudan and Ethiopia, and classified them into 15 genotypes (named A-O), although the majority of Portuguese samples belonged to two genotypes (A and B). Later, the same technique was used by da Silva et al. (2015) to evaluate the genetic variability of *L. infantum* in humans and dogs from Brazil. Results revealed *L. infantum* genetic homogeneity with all samples grouping in genotype B, which agreed with the results of Cortes et al. (2006) in Brazilian samples. Nevertheless, Silva et al. (2015) discovered a new pattern, not previously described by Cortes et al (2006), from a GenBank reference sequence (AF169133) which came from an Algerian *L. infantum* sequence and was called “New genotype”. These studies confirm kDNA-PCR-RFLP as a valid methodology to evaluate *L. infantum* intraspecific variability. Rotureau et al. (2006) also found a variable degree of intraspecific polymorphisms in all species.

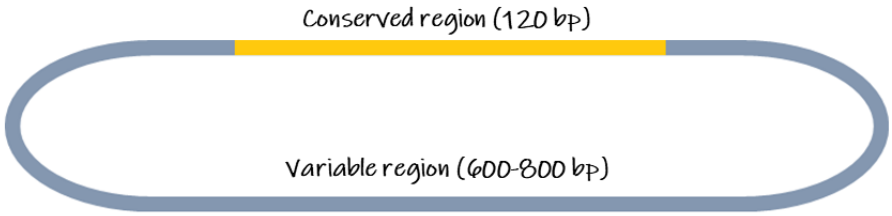

Figure 5. Conserved and variable regions of *Leishmania* spp. kinetoplast minicircle. Based on der Auwera & Dujardin, 2015.

Concerning other genes, in the study of Fernández-Arévalo et al. (2021), hsp70 and ITS2 were able to detect several variants within a particular zymodeme for *L. major, L. guyanensis* and *L. braziliensis* complexes. The mini-exon is a region with one to two hundred copies per genome and specific of kinetoplastids, and the amplified products vary in size according to *Leishmania* complex and even within the same species (Marfurt et al., 2003). In the latter study, no difference was found between *L. chagasi* and *L. infantum*, as they are considered synonym species (Maurício et al., 2000). It also highlighted a close phylogenetic relationship between *L. major* and *L. venezuelensis*, which was also later described by Berzunza-Cruz et al. (2002) using other markers, suggesting that *L. major* populations were imported from the Old World to the Americas (Marfurt et al., 2003). Finally, hsp20 or hsp70 have been used to investigate phylogenetic relationships between *Leishmania* species. Fraga et al. (2013) identified up to 9 species and 2 subspecies with these markers. These genes have also been used with PCR-RFLP, but it presents some specificity limitations, as it also amplifies *Trypanosoma* spp.

Sandfly salivary antigens as a marker of sandfly exposure

Sandfly saliva is compounded by several antigenic proteins that trigger an immunological response in the vertebrate host known to influence *Leishmania* spp. ability to stablish an infection (Rohousova & Volf, 2006). Serological methods targeting anti-saliva antibodies have been developed to measure exposure to sandfly bites and the risk for *Leishmania* and other sandfly-borne pathogens' transmission.

Salivary proteins are species-specific and sialome composition varies between sandfly species (Volf & Rohousová, 2001), but it was similar between populations of the same species from different origins (Martín-Martín et al., 2012). In addition, the immune response to each of the salivary antigenic proteins also differs depending on the bitten host

species and even at an individual level (Martín-Martín et al.,2012; Barral et al., 2000). Pioneering studies used sandfly salivary gland homogenates (SGH) as an antigen source in serological assays. However, the difficult procurement of SGH which requires individual sandfly dissection, the difficulty in standardising preparations and the limited species specificity in some cases, triggered research to identify and produce recombinant salivary proteins (rSP). Whilst they are indeed easier to obtain and to standardise, only a few have proved to be useful in large scale epidemiological studies. Studies in dogs show that the family of the yellow protein rSP03B have the best antigenic properties for serological diagnosis (Kostalova et al., 2017; Martín-Martín et al., 2014; Risueño et al., 2019).

L. infantum coinfection with other pathogens

Pathogenic agents including parasites, viruses and bacteria commonly co-exist with *L. infantum* in the same host. This is particularly the case in areas where the infectious agents and their vectors are plentiful, and if they are favoured by the same environmental conditions and host immunological mechanisms. Moreover, climate change can enhance the distribution of vectors and VBI, as is the case with sandflies and *L. infantum*, which are expanding towards previously non-endemic areas further north and at higher altitudes (Ballart et al., 2012; Martín-Sánchez et al., 2009; Ready, 2010).

Co-infections and co-seropositivities have been described for *L. infantum* and *Toxoplasma gondii, Neospora caninum, Ehrlichia* spp., *Anaplasma* spp., *Rickettsia rickettsii, Dirofilaria immitis, Borrelia burgdorferi* (Oliveira et al., 2021; Baxarias et al., 2018; Tabar et al., 2013; Cringoli et al., 2002). However, there is scarce information concerning the pathogenic mechanisms and clinical implications of coinfections between *L. infantum* and other pathogens. There is good evidence, that immunosuppressive infections such as HIV in humans is a major risk factor for developing clinical leishmaniosis (Alvar et al., 1997; Alvar et al., 2008; Singh, 2014) and, in coinfected patients, viral and parasitic loads in blood are positively correlated (Colomba et al., 2009). Also, Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) have been found associated with *L. infantum* infection in cats (Sherry et al., 2011; Ayllón et al., 2012; Iatta et al., 2019; Pennisi et al., 2012), although the role of these retroviruses in FeL is still in debate. Other coinfections potentially associated to FeL are Feline Coronavirus (FCoV), "*Candidatus Mycoplasma turicensis*" and *Hepatozoon* spp. (Attipa et al., 2017; Spada et

al., 2016). In the study of Montoya-Alonso et al. (2020), coinfections between different vector-borne pathogens were detected in 15% of dogs, and the risk of *L. infantum* infection increased significantly in the case of coinfections with *Anaplasma* spp. and *Ehrlichia canis*, and vice versa. Similarly, other reports provide evidence that coinfections with vector-borne pathogens (*Ehrlichia canis*, *Anaplasma* spp., *Neospora caninum* and *Dirofilaria immitis*) increased dog's susceptibility to CanL and vice versa (Baxarias et al., 2018; Cringoli et al., 2002; Paulan et al., 2013; Tabar et al., 2013; Toepp et al., 2019; Zulpo et al., 2012). However, the synergic immunological mechanisms of different VBI are still unknown. Also, the potential interaction between *Leishmania* parasites and sandfly-borne phleboviruses raises much interest but no evidence of the implications of this coinfection have been provided yet (Alkan et al., 2013). Maia et al. (2017) reported an association between *L. infantum* and Sicilian virus (SFSV) in dogs from Portugal, and in the study of Pereira et al. (2019), FeL-compatible clinical signs were associated to exposure to SFSV.

Phlebovirus infection in human hosts

Human phleboviroses are caused by RNA stranded viruses belonging to *Phlebovirus* genus, family Phenuiviridae. They include at least 67 species some of them with more than one antigenic variant, due to their high mutation rate (Calisher & Calzolari, 2021). They are divided in two main groups: those transmitted by sandflies and those by ticks and, in sandflies, both vertical and venereal transmission have been proved (Tesh et al., 1992). The geographical distribution of sandfly-borne phleboviruses includes most of southern European countries, where at least 16 species including several variants have been identified (Table 3), and Africa, the Indian subcontinent, the Middle East and Central Asia (Alkan et al., 2013; Alwassouf et al., 2016a; Ayhan & Charrel, 2018a, b; Davó et al., 2020).

Phlebovirus induce a strong humoral response in the host. In Spain, antibodies against SFSV, Naples (SFNV), Granada (GRV) and Toscana (TOSV) phleboviruses have been detected in humans. Only TOSV is considered to cause severe neurological human illness, and the other phleboviruses are implicated in mild syndromes (Collao et al., 2010; Echevarría et al., 2003; Martínez-García et al., 2007; Mendoza-Montero et al., 1998; Navarro-Marí et al., 2013). In addition, Massilia, Arbia and Arrabida viruses have been detected in Spain in sandflies only (Davó et al., 2020; Remoli et al., 2016; Sánchez-Seco et al., 2010). Antibodies can be detected by several assays including seroneutralization, ELISA and IFAT tests. The virus seroneutralization test is the most sensitive and specific

and the reference antibody test, whilst ELISA and IFAT are sensitive but have low specificity as cross-reactions have been reported between antigenically related phleboviruses (Alkan et al., 2013). In Spain, human TOSV seroprevalence ranges from 5 to 26% and increases with age (Cardeñosa et al., 2013; Navarro et al., 2004). Toscana virus infections are commonly asymptomatic or mild causing flu-like symptoms and, sporadically, patients develop meningitis, encephalitis, and other severe neurological complications (Ayhan & Charrel, 2020; Sanbonmatsu-Gámez et al., 2005). Phlebovirosis is more common in people living in rural areas and it is usually present during the sandfly season, from May to October (Navarro et al., 2004). The overall clinical impact of TOSV in Spain is low and very low for other phleboviruses (García San Miguel et al., 2020). However, several studies in southern Europe show that TOSV prevalence is increasing, and it is considered an emergent pathogen (Charrel et al., 2012).

Table 3. *Phlebovirus* species in European countries.

Phlebovirus species	Country	Host
Adana	Cyprus	Dogs
Adria	Italy, Greece, Albania	Humans, sandflies
Alcube	Portugal	Sandflies
Arbia	Italy, Spain, Greece, Cyprus	Sandflies, dogs
Arrabida	Portugal, Spain	Sandflies
Balkan	Albania, Bosnia & Herzegovina, Croatia	Sandflies
Bregalaka	Macedonia	Sandflies
Corfu	Greece	Humans, sandflies
Cyprus	Cyprus	Humans
Granada	Spain	Humans, sandflies
Karimabad	Moldova, Azerbaijan	Humans
Massilia	Spain, France, Portugal	Sandflies
Naples	Italy, Greece, Croatia, Bosnia & Herzegovina, Serbia, Malta, Cyprus, Moldova, Azerbaijan	Humans, sandflies
Sicilian	Italy, France, Greece, Croatia, Cyprus, Bosnia & Herzegovina, Moldova, Azerbaijan	Humans, sandflies, sheep, deer, cattle, dogs
Toscana	Italy, France, Spain, Portugal, Greece, Croatia, Bosnia & Herzegovina, Malta, Cyprus	Humans, sandflies, dogs, cats, goats, sheep, cows, pigs, horses
Zaba	Croatia	Sandflies

As mentioned, our understanding of the epidemiology of *Phlebovirus* infections is scarce and no reservoirs of infections have been identified yet, although antibodies against the some phleboviruses have been detected in many domestic species. For example, there is serological evidence of TOSV and SFSV infections in horses, dogs, cats, sheep, pigs,

goats and cows from Europe (Alwassouf et al., 2016a, b; Ayhan et al., 2017; Navarro-Marí et al., 2011).

Since both phleboviruses and *L. infantum* are transmitted by sandflies, an epidemiological link between them was suggested. This association was confirmed for TOSV and *L. infantum* in humans from France (Bichaud et al., 2011) and for SFSV and *L. infantum* in dogs from Portugal (Maia et al. 2017).

Babesia spp. and *Hepatozoon* spp. infections in animal hosts

Babesia spp. and *Hepatozoon* spp. are tick-borne apicomplexan protozoans infecting the erythrocytes and leukocytes, respectively, of animals and humans. Whilst *Babesia* parasites are transmitted by a tick bite, in the case of *Hepatozoon* spp. transmission occurs mostly when the tick is ingested by the host. However, other transmission routes have been demonstrated for certain parasite species, such as such as transplacentally in the case of *Hepatozoon canis* (Murata et al., 1993), by predation for *H. americanum* (Johnson et al., 2009), through blood transfusion for *Babesia* spp., (Baneth, 2011) and dog-to-dog transmission through fighting in the case of *Babesia. gibsoni* (Birkenheuer et al., 2005). Both genera comprise several species among which some are specific for a determined host species, and only a few of them present zoonotic potential (Springer et al., 2020). Infection prevalences from large epidemiological studies in dogs and cats from Europe are, respectively, 0-48% and 0-26% for *Hepatozoon* spp., and 0-50% and 0-8% for *Babesia* spp. Also, *Hepatozoon* spp. and *Babesia* spp. prevalences can reach over 90% in some wild carnivore species like foxes, badgers or martens (Battisti et al., 2020; Cimpan et al., 2020; Hamel et al., 2012; Javier Millán et al., 2016; Morelli et al., 2021; Movilla et al., 2017; Ortuño et al., 2021; Otranto et al., 2017; Vilhena et al., 2013). The clinical manifestations of babesiosis derive from the hemolysis, release of hemolytic toxins and anemia caused by the parasites, and disease severity depends on the infecting *Babesia* species (Lempereur et al., 2017).

The risk of infection with these parasites depends on animals' exposure to the widely distributed tick vectors, although there are other alternative transmission routes for some apicomplexan species. Also, *Babesia* spp. and *Hepatozoon* spp. have been detected in *L. infantum*-infected animals and *vice versa* (Guadalupe Miró et al., 2015; M. Tabar et al., 2008, 2009; Vilhena et al., 2013) but there are only two studies reporting an association

between *Hepatozoon* spp. and *L. infantum* infections in cats (Attipa et al., 2017) and in wild carnivores (Ortuño et al., 2021).

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OBJECTIVES

The **general aim** of this doctoral thesis is to deepen our understanding of some aspects of the epidemiology of the sandfly-borne *Leishmania infantum* and phleboviruses and tick-borne *Hepatozoon* spp. and *Babesia* spp. protozoan parasites in animals and humans from southern Spain. The specific objectives are:

1. To estimate prevalence, associations and clinical features of *Leishmania infantum*, *Hepatozoon* spp. and *Babesia* spp. infections in cats and dogs. This objective is described in **Chapter 1.**

2. To estimate the prevalence and associations between *Leishmania infantum*, *Hepatozoon* spp. and *Babesia* spp. in wildlife. This objective is presented in **Chapter 2.**

3. To estimate prevalence and associations between *Leishmania infantum* and Toscana and Sicilian phleboviruses, and the degree of exposure to the vector *Phlebotomus perniciosus* in blood donors. This objective is described in **Chapter 3.**

4. To analyse genetic variability of *Leishmania infantum* in humans and animals to improve knowledge of its aetiology, relationship with demographic and clinical variables and the potential for interactions between *Leishmania* spp. transmission between humans, dogs and wildlife and generally, between domestic and sylvatic transmission environments. This objective is presented in **Chapter 4.**

CHAPTER 1

Clinical, diagnostic and epidemiological implications of *Leishmania infantum*, *Hepatozoon* spp. and *Babesia* spp. infection in cats and dogs in a Mediterranean periurban ecosystem

Abstract

The kinetoplastid *Leishmania infantum* and apicomplexans *Hepatozoon* spp. and *Babesia* spp. are common in dogs and cats in Mediterranean ecosystems, and a study in Spain indicated that *L. infantum* infected wildlife were more likely to be infected with the apicomplexans. Knowledge of *L. infantum* infection in cats is scarce compared to dogs. We used PCR and DNA sequencing to investigate the prevalence of the three parasites and aetiology of *Hepatozoon* spp. and *Babesia* spp., in blood, skin and lymphoid tissue from 212 stray cats and 82 abandoned dogs in southeast Spain. *Leishmania infantum* DNA was detected in 44% of dogs and 21% of cats, *Hepatozoon felis* in 25% of cats, *H. canis* in 13% of dogs, and *Babesia vogeli* in one dog. *Hepatozoon* spp. were identical to those previously found in wildlife, suggesting a common transmission cycle, but it was not associated to *L. infantum* infection. *L. infantum* prevalence was higher in skin and lymphoid tissue than in blood in healthy cats and dogs, and similar in cats with clinical signs. Prevalence in lymphoid tissue was lower in cats compared to dogs, and median CTs were higher in cats than in dogs, in healthy cats compared to those with clinical signs, and in young compared to older cats ($p<0.05$). Results suggest that infection in cats is less likely to affect lymphoid tissue than in dogs and when it does, it is associated with lower parasite burdens. This is consistent with cats' lower risk of leishmaniosis and having mostly cutaneous lesions.

Introduction

Dogs and cats harbour several vector-borne pathogens and the incidence of these infections is considered to be increasing (Wright et al., 2020). They are an integral part of society and preserving their health is crucial to ensure their welfare and prevent transmission of zoonotic pathogens to humans. The kinetoplastid protozoan *Leishmania infantum,* transmitted by phlebotomine sand flies, is a relevant veterinary problem with major Public Health implications in Mediterranean countries. The parasite targets reticuloendothelial cells and infects a wide range of vertebrate species. Whilst dogs are the most susceptible species and the main domestic reservoir of infection, other domestic and wild animal species may develop disease and become parasite reservoirs. Xenodiagnostic experiments have demonstrated cat's ability to transmit infection to vectors (Maroli et al., 2007; Mendonça et al., 2020) and feline leishmaniosis (FeL) is being increasingly reported

worldwide (Abramo et al., 2021). *Leishmania infantum* was recently detected in a wild cat and other wildlife species in southern Spain and an association was found between infections with *L. infantum* and *Hepatozoon* spp. and *Babesia* spp. (Ortuño et al., 2021). The latter tick-borne apicomplexan protozoan parasites of vertebrates, display a higher degree of host specificity and a comparatively low zoonotic potential (Springer et al., 2020). *Babesia* spp. infects erythrocytes and *Hepatozoon* infects leukocytes and the severity of babesiosis and hepatozoonosis depends on the host species and it may range from asymptomatic infections to severe illness (Baneth et al., 2007; Irwin, 2010; Solano-Gallego & Baneth, 2011).

The present study builds on the previously described work on leishmaniosis, hepatozoonosis and babesiosis in wildlife in southern Spain with the following aims: (i) estimating *L. infantum* prevalence in domestic stray cats by PCR analysis of blood, skin and lymphoid tissue samples, (ii) assessing the etiology and prevalence of *Hepatozoon* spp. and *Babesia* spp. in stray cats and abandoned dogs by PCR and DNA sequence analysis and (iii) investigating the statistical relationship between infections by the three parasites. We expected to provide clinical, diagnostic and epidemiological information on these infections in dogs and cats and on the extent to which *Hepatozoon* spp. and *Babesia* spp. species are shared between domestic animals and wildlife.

Materials and methods

Study population and experimental design

Animals included 212 stray cats and 82 abandoned dogs from the city of Murcia (Spain) metropolitan area collected by the local authorities between 2010 and 2016 as part of a zoonosis control program. Animals in this program are clinically examined, and those that cannot be rehomed were eventually euthanised. Those included in this study were among the latter group and were randomly selected. Clinical signs were detected in 53% (112/212) cats and 2% (2/82) of dogs. In cats, they included low body condition (69/112), lymphadenomegaly (34/112), cutaneous lesions (32/112), diarrhoea (20/112), oral lesions (14/112), ocular signs (6/112), respiratory signs (4/112), jaundice (1/112) and pale mucous membranes (1/112). Whilst a formal post-mortem examination was not performed, splenomegaly and/or spleen lesions were detected in 10/112 cats when samples from this

organ were collected for *L. infantum* diagnosis. Clinical signs in dogs were alopecia, ulcerative dermatitis, lymphadenomegaly and muscular atrophy in one dog, and pale mucous membranes and spleen lesions in the other dog. Animals were grouped according to age as young (<3 years-old) or adult (>3 years-old), and by size as small, medium and big based on weight (in dogs: <10 kg, 10-20 kg and >20 kg, respectively; in cats: ≤ 3 kg, 3-4.5 kg and >4.5 kg, respectively).

Hepatozoon spp. and *Babesia* spp. infections were analysed by PCR in whole blood samples from 123 cats and all 82 dogs. *Leishmania* was investigated in blood, skin and lymphoid tissue samples including spleen and lymph node, from 212 cats and 82 dogs. Data on the dog's *Leishmania* PCR were previously reported (Ortuño et al., 2017; Risueño et al., 2012) and were used here to analyse coinfections between parasites.

DNA extraction and PCR amplification of *Babesia* spp., *Hepatozoon* spp. and *L. infantum* DNA

Dog and cat DNA were purified using a robot (Maxwell® Promega, Madison, WI, USA), and a commercial kit (Extractme Genomic DNA kit, Blirt©), respectively. Apicomplexan infection was firstly assessed by a generic end-point PCR amplifying the 18S ribosomal RNA (rRNA) (Tabar et al. 2008; Margalit Levi et al., 2018). Positive samples were further analysed by a second PCR targeting the same region, this time with specific primers for *Babesia* spp. (Barbosa et al., 2020; Olmeda et al., 1997) and *Hepatozoon* spp. (Almeida et al., 2012; Sarma et al., 2019). Positive control DNA was obtained from *Babesia* spp. or *Hepatozoon* spp. naturally infected dogs. DNA from a non-infected dog was used as negative control, and ultra-pure water was employed as non-template DNA control.

L. infantum DNA from cat samples was amplified by a real-time PCR (rtPCR) assay with a Taqman probe targeting kinetoplast minicircle DNA (kDNA) (Dantas-Torres et al., 2017). Dogs had been previously tested using a similar TaqMan kDNA-rtPCR (Mary et al. 2004). Samples were deemed PCR-positive for cycle thresholds (CT) ≥38 (Mackay, 2007).

Babesia and *Hepatozoon* DNA sequencing and phylogenetic analyses

Babesia spp. and *Hepatozoon* spp. positive PCR products were sequenced and the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) used to compare with reference

sequences in GenBank. They are now available in the GenBank database under the accession numbers MZ424831-7.

Phylogenetic trees were constructed using the MEGA X software (Kumar et al., 2018) and tree topology was inferred using the Neighbour-Joining method. Tree branches showing bootstrap values >70% were considered confident. Homologous sequences of *Plasmodium falciparum* (accession number MF155937) and *Sarcocystis cymruensis* (MG564723) were used as outgroup in *Babesia* spp. and *Hepatozoon* spp. phylograms, respectively.

Statistical analysis

Fisher's exact and the non-parametric Kruskal-Wallis tests were used to compare PCR prevalence (% positives) and median CTs of *L. infantum*-PCR positive animals, respectively, according to demographic and clinical variables. Since poor body condition in stray cats may be related to several reasons, this clinical sign was not included in the analyses. For some comparisons, animals PCR-positive in more than one sample type were assigned the CT of the sample with the lowest value. The degree of agreement between PCR tests in different biological samples was analysed with the kappa statistic (Thrusfield, 2018). Associations were considered statistically significant for $p<0.05$. The R software (http://cran.r-project.org/) was used for all analysis.

Results

Babesia spp., *Hepatozoon* spp. and *L. infantum* infection PCR prevalences and relationship with explanatory variables

The percentage of PCR-positive animals were: for *Babesia* spp., 0% (0/123) in cats and 1% (1/82) in dogs; for *Hepatozoon* spp., 25% (31/123) in cats and 13% (11/82) in dogs and, for *L. infantum*, 21% (44/212) in cats and 44% (36/82) in dogs (Table 1). There were no significant associations between parasitic infections. Mixed *Hepatozoon* spp. and *L. infantum* infections were detected in 6% (12/205) of the animals (7% in dogs and 5% in cats) and no animal was infected with the three parasites.

Table 1. Number (%) of *Babesia* spp., *Hepatozoon* spp. and *Leishmania infantum* PCR-positive dogs and cats.

	B. vogeli	***H. canis***	***H. felis***	***L. infantum***						
				B+Sk+ Sp+LN	**Blood**	**Sk+Sp+ LN**	**Skin**	**Lymphoid tissue**		
								All	**Spleen**	**LN**
Cats	0/123 (0)	0/123 (0)	31/123 (25)	44/212 (21)	7/123 (6)	43/176 (24)	24/147 (16)	31/174 (18)	28/153 (18)	3/22 (14)
With clinical signs	0/43 (0)	0/43 (0)	12/43 (28)	15/81 (19)	6/43 (14)[a]	15/75 (20)	9/65 (14)	13/74 (18)	11/65 (17)	2/9 (22)
Without clinical signs	0/79 (0)	0/79 (0)	19/79 (24)	28/130 (22)	1/79 (1)[a]	27/100 (27)	14/81 (17)	18/100 (18)	17/87 (20)	1/13 (8)
Dogs	1/82 (1)	11/82 (13)	0/82 (0)	36/82 (44)	3/76 (4)	36/82 (44)	8/65 (12)	34/82 (41)	27/82 (33)	14/64 (22)

[a] Significant differences $p<0.05$; B – blood; Sk – skin; Sp – spleen; LN – lymph node

L. infantum prevalence differed according to the sample type and host species. In cats, it was 6% (7/123) in blood, 14% (3/22) in lymph node, 16% (24/147) in skin and 18% (28/153) in spleen. In dogs, it was 4% (3/76) in blood, 12% (8/65) in skin, 22% (14/64) in lymph node and 33% (27/82) in spleen. Prevalence was higher in skin and lymphoid tissue compared to blood in dogs and cats, in dog lymphoid tissue compared to cat lymphoid tissue, in blood from symptomatic compared to asymptomatic cats and in animals sampled in the winter compared to other times ($p<0.05$) (Tables 2 and 3). Median (range) CTs in PCR-positives were 32 (12-38) in dogs and 35 (13-38) in cats, and were lower in lymphoid tissue from dogs than in the same tissue in cats, in cats with symptoms compared to those without and in older compared to younger cats ($p<0.05$) (Table 3). Prevalence in cats was not associated to specific clinical sign or poor body condition ($p>0.05$).

Kappa statistic reflected only slight, fair or moderate agreement between PCR results in different types of samples. All cats and dogs positive in blood were positive in skin, spleen and lymph node, except one dog positive in blood and not in skin. In contrast, skin and lymphoid tissue samples complemented each other, so for example, only half of cats positive in skin were also positive in spleen and vice versa (Table 2).

Table 2. P-values resulting from the comparison of *L. infantum*-positive animals in different samples (p) and *kappa* index (k) and degree of agreement in those animals tested simultaneously in different samples.

		Cat				**Dog**			
		Blood	**Skin**	**Spleen**	**Lymph node**	**Blood**	**Skin**	**Spleen**	**Lymph node**
Cat	**Blood**	-	Fair	Fair	Moderate	-	-	-	-
	Skin	p=0.011 k=0.23	-	Fair	NA	-	-	-	-
	Spleen	p=0.003 k=0.30	p=0.765 k=0.40	-	NA	-	-	-	-
	Lymph node	p=0.369 k=0.60	p=0.993 k=NA	p=0.813 k=NA	-	-	-	-	-
Dog	**Blood**	p=0.8312	-	-	-	-	Slight	Slight	Fair
	Skin	-	p=0.585	-	-	p=0.126 k=0.16	-	Fair	Moderate
	Spleen	-	-	p=0.018	-	p<0.001 k=0.19	p=0.007 k=0.27	-	Moderate
	Lymph node	-	-	-	p=0.598	p=0.003 k=0.21	p=0.226 k=0.46	p=0.198 k=0.42	-

The prevalence of *Hepatozoon* spp. infection in cats was similar in symptomatic compared to asymptomatic animals, but marginally higher in those with poor body condition (Table 4) and in young compared to adult cats (Table 3) ($p<0.10$), and greatest in the summer ($p<0.05$). In contrast, in dogs, *Hepatozoon* spp. infection was highest in the winter ($p<0.05$), but no samples were collected in the summer (Table 3).

The *Babesia* spp. infected dog was an asymptomatic, 4-years old female Chow-Chow sampled in spring.

Table 3. Prevalence (%) of *Hepatozoon* spp. and *L. infantum* infection and median (range) *L. infantum* CTs in dogs and cats from Murcia, according to demographic variables and clinical status.

		Dogs							**Cats**							
		H. canis			***L. infantum*[2]**				***H. felis***			***L. infantum***				
Variable	**Level**	**No.**	**% +ve[1].**	***P* value**	**% +ve.**	***P* value**	**Median CT (range)**	***P* value**	**No.**	**% +ve.**	***P* value**	**No.**	**% +ve.**	***P* value**	**Median CT (range)**	***P* value**
Sex	Female	35	11	0.7492	46	0.8213	32 (16-38)	0.6033	58	22	0.5353	106	19	0.6118	33 (27-38)	0.5898
	Male	46	15		41		30 (12-38)		64	28		105	22		36 (13-38)	
Age	Young	30	10	1	30	0.1570	30 (14-35)	0.4820	58	33	0.0956	108	18	0.3097	38 (29-38)	0.0004
	Adult	48	10		48		32 (12-38)		65	18		104	24		31 (13-38)*	
Breed	Pure	36	14	0.7446	42	0.8222	32 (12-38)	0.6156	120	26	0.5712	204	20	0.3670	36 (13-38)	0.3130
	Crossed	45	11		47		32 (14-36)		3	0		8	38		31 (31-35)	
Weight	Small	23	4	0.5903	30	0.3724	32 (24-35)	0.8438	47	23	0.7887	99	16	0.0698	36 (27-38)	0.4576
	Medium	21	10		33		28 (16-38)		63	25		95	21		34 (13-38)	
	Big	22	14		50		32 (22-36)		12	33		17	41		31 (19-38)	
Collection season	Spring	49	10	0.0163	33	0.0032	32 (12-35)	0.7436	1	0	0.0419	59	2	<0.0001	36 (36-36)	0.1670
	Summer	0	-		-		-		27	44*		51	16		31 (21-38)	
	Autumn	20	5		45		31 (16-38)		58	24		58	9		38 (32-38)	
	Winter	13	38*		85*		34 (14-38)		35	14		37	73*		35 (13-38)	
Clinical status	Symptomatic	2	50	0.2330	100	0.1944	16 (12-20)*	0.0270	43	22	0.6675	81	19	0.7256	30 (13-38)*	0.0133
	Asymptomatic	79	11		43		32 (14-38)		79	24		130	22		36 (27-38)	
Leishmania spp. infected	Yes	36	17	0.5227	-	-	-	-	37	16	0.1753	-	-	-	-	-
	No	46	11		-		-		86	29		-	-		-	
Hepatozoon spp. infected	Yes	11	-	-	55	0.5227	23 (20-38)	0.5678	31	-	-	31	19	0.1753	38 (21-38)	0.2119
	No	71	-		42		27 (12-38)		92	-		92	34		30 (13-38)	

[1] PCR-positive

[2] Data of *L. infantum* infection in dogs (N=82) from southeast Spain was published previously (Risueño et al., 2012; Ortuño et al., 2017) and included here to allow comparisons.

Table 4. Number (percentage) of cats presenting different clinical signs according to *L. infantum* and *H. felis* infection status[1].

Clinical sign	*L. infantum*		*H. felis*	
	Positive	**Negative**	**Positive**	**Negative**
Poor body condition	3/43 (7)	66/168 (39)	13/31 (42)[a]	21/91 (23)[a]
Lymphadenomegaly	8/43 (19)	26/168 (15)	6/31 (19)	15/91 (16)
Cutaneous lesions	8/43 (19)	24/168 (14)	7/31 (23)	15/91 (16)
Exfoliative dermatitis	0/43 (0)	2/168 (1)	0/31 (0)	2/91 (2)
Ulcerative dermatitis	3/43 (7)	1/168 (1)	1/31 (3)	3/91 (3)
Scabs	1/43 (2)	5/168 (3)	1/31 (3)	3/91 (3)
Alopecia	0/43 (0)	1/168 (1)	0/31 (0)	1/91 (1)
Eczema	0/43 (0)	2/168 (1)	1/31 (3)	1/91 (1)
Wounds	6/43 (14)	15/168 (9)	5/31 (16)	9/91 (10)
Diarrhoea	6/43 (14)	14/168 (8)	1/31 (3)	8/91 (9)
Oral lesions	2/43 (5)	12/168 (7)	1/31 (3)	3/91 (3)
Tongue ulcers	1/43 (2)	3/168 (2)	0/31 (0)	2/91 (2)
Gingivitis	1/43 (2)	10/168 (6)	1/31 (3)	1/91 (1)
Splenomegaly/spleen lesions	0/43 (0)	10/168 (6)	2/31 (6)	3/91 (3)
Ocular lesions	2/43 (5)	4/168 (2)	1/31 (3)	3/91 (3)
Conjunctivitis	1/43 (2)	2/168 (1)	1/31 (3)	1/91 (1)
Corneal ulcers	1/43 (2)	1/168 (1)	0/31 (0)	2/91 (2)
Keratitis	1/43 (2)	2/168 (1)	0/31 (0)	2/91 (2)
Hyphema	1/43 (2)	0/168 (0)	0/31 (0)	1/91 (1)
Respiratory signs	1/43 (2)	3/168 (2)	0/31 (0)	1/91 (1)
Jaundice	0/43 (0)	1/168 (1)	0/31 (0)	0/91 (0)
Pale mucous membranes	0/43 (0)	1/168 (1)	0/31 (0)	1/91 (1)

[1] Clinical data from one cat was missing
[a] Marginally significant differences $p<0.10$

Characterization and phylogenetic analyses of *Babesia* spp. and *Hepatozoon* spp. DNA sequences

The *Babesia* spp. sequence identified was 100% identical to *B. vogeli* (accession no. MK910150). Three different *Hepatozoon* spp. sequences differing 1 to 3 nucleotides were obtained from 11 PCR amplicons from dogs and were 100% identical to *H. canis* reference sequences (MN628317, MN628318, MN628320), and were named HC16, HC17 and HC18. Similarly, three *Hepatozoon* spp. sequences differing 1 to 3 nucleotides, from 31 PCR amplicons from cats were 99.7-100% identical to *H. felis* (MG386483, MG386484) and coded HF4, HF5 and HF6 (Table 5).

Table 5. Results of the BLAST analysis for the *Babesia* spp. and *Hepatozoon* spp. positive samples.

New GenBank Acc. No.	Isolate*	Parasite species	Host (N)	Seq. length (bp)	Closest GenBank Acc. No.	Identity/ Query cover (%)
MZ424831	BVo1	*Babesia vogeli*	Dog (1)	284	MK910150	100/100
MZ424832	HC16	*Hepatozoon canis*	Dog (5)	337	MN628317	100/100
MZ424833	HC17	*Hepatozoon canis*	Dog (2)	337	MN628318	100/100
MZ424834	HC18	*Hepatozoon canis*	Dog (4)	337	MN628320	100/100
MZ424835	HF4	*Hepatozoon felis*	Cat (29)	337	MG386483	100/100
MZ424836	HF5	*Hepatozoon felis*	Cat (1)	337	MG386484	100/100
MZ424837	HF6	*Hepatozoon felis*	Cat (1)	337	MG386484	99.7/100

* Isolate names are consecutive to those published in wildlife from Spain by Ortuño et al. (2021).

In the phylogenetic analyses, *B. vogeli* from the dog (isolate BVo1) clustered with those in dogs from France, Malta, Colombia, Algeria and China, cats from Qatar, China and Brazil and foxes from France (99% bootstrap) (Fig. 1).

H. canis isolate HC16 was in the same cluster as isolates HC1 and HC9 from foxes in southern Spain including Murcia region, and also with *H. canis* found in foxes from France and in dogs from Algeria and India (71% bootstrap) (Fig.2). Isolates HC17 and HC18 clustered with *H. canis* from dogs in Spain, Tunisia, Taiwan, India and Israel, cats and golden jackals from Israel, foxes from Murcia (HC2) and a beech marten from Andalucía (HC3), Spain (70% bootstrap) (Fig. 2).

H. felis isolate HF4 clustered with *H. felis* from domestic cats in Spain, Israel, Italy and Angola (86% bootstrap), and a wild cat from Andalucía, Spain (HF1). Isolates HF5 and HF6 grouped with *H. felis* found in cats from Italy and Angola and in wild cats from Bosnia and Herzegovina, and also with *H. ingwe* from an African leopard from South Africa, and separate from isolate HF2 found in wild cats from Murcia (Fig. 2).

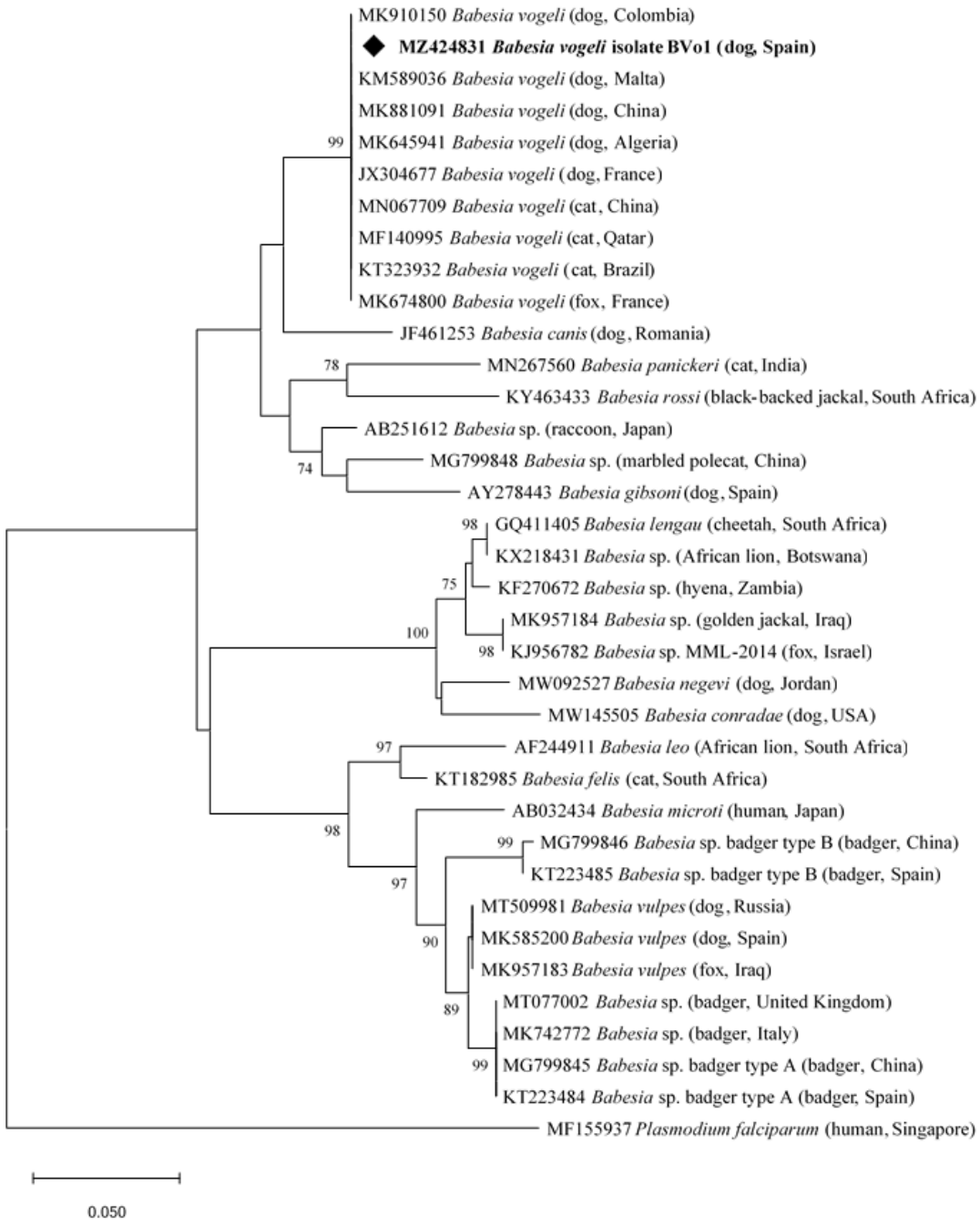

Figure 1. Phylogenetic tree of *Babesia* spp. sequences from dogs and cats in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Neighbour-Joining method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 275 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

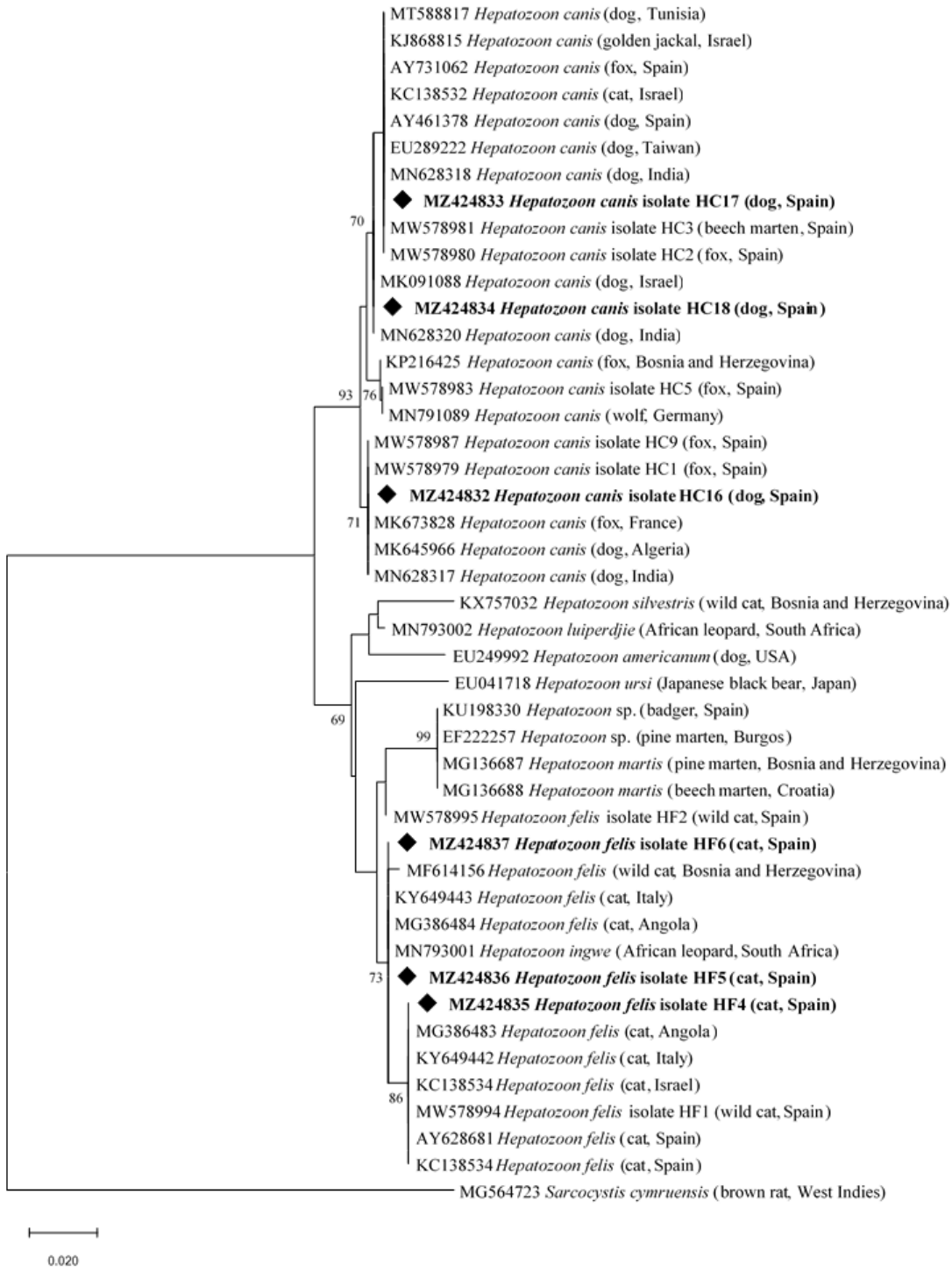

Figure 2. Phylogenetic tree of *Hepatozoon* spp. sequences from dogs and cats in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Neighbour-Joining method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 334 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Discussion

The *Hepatozoon* spp. DNA sequences in abandoned dogs and stray cats were in most cases identical to those in foxes, beech martens and wild cats in southern Spain (Ortuño et al., 2021), suggesting a common transmission cycle, as postulated for *L. infantum* (Ortuño et al., 2019). In contrast Ortuño et al. (2021) and cats in Cyprus (Attipa et al., 2017), *Hepatozoon* spp. infection was not associated with *L. infantum*. Potential synergism between leishmaniosis and other VBI in dogs and cats has been postulated (Baxarias et al., 2018; Cringoli et al., 2002; Mekuzas et al., 2009; Toepp et al., 2019), but the pathogenesis of apicomplexan parasites and *Leishmania* coinfections have not been stablished.

H. canis and *B. vogeli* are transmitted by *R. sanguineus*, the most common tick in dogs in Spain (Checa et al., 2019; Estrada-Peña et al., 2017). The almost absence of *Babesia* spp. in dogs and cats suggests a low circulation of these parasites in the studied area and not because of low exposure to *R. sanguineous* since *H. canis* was found in 13% of dogs. Other articles have similarly reported low prevalences of *B. canis*, *B. vogeli* and *B. gibsoni* in dogs in Spain (Baxarias et al., 2018; Movilla et al., 2017; Tabar et al., 2009), whereas *B. vulpes* in dogs and foxes is more prevalent in northwest Spain and Portugal (Checa et al., 2019; García, 2006; Miró et al., 2015). Feline babesiosis is rare and mostly restricted to South Africa (Jacobson et al., 2000). Vilhena et al. (2013) found 11% of *B. vogeli* PCR-positive cats in Portugal and *Babesia* spp. positive cats have been sporadically reported elsewhere in Europe and Asia (Penzhorn & Oosthuizen, 2020).

The prevalence of *H. canis* in dogs and *H. felis* in cats reported here is higher than most studies in the Iberian Peninsula where it ranged from 0.7% to 3.3% for *H. canis* and 1.4% to 16% for *H. felis* (Díaz-Regañón et al., 2017; Maia et al., 2015; Movilla et al., 2017; Ortuño et al., 2008; Tabar et al., 2009; Vilhena et al., 2013). Albeit, Criado-Fornelio et al. (2006) and Dordio et al. (2021) reported 20% and 27% *H. canis* and *H. felis* prevalence, respectively. The prevalence of *H. canis* and *H. felis* in other European countries was similarly variable (Cimpan et al., 2020; Licari et al., 2017; Otranto et al., 2017). In contrast to other studies from Spain and other countries in Europe (Criado-Fornelio et al., 2009; Díaz-Regañón et al., 2017; Giannelli et al., 2017), we did not detect *H. canis* or *H. silvestris* in cats.

The risk of infection with *Hepatozoon* spp. depends on exposure to ticks and is conditioned by the animal's lifestyle, use of ectoparasiticidals and contact with wildlife

(Pacifico et al., 2020). It's likely that most of the animals in this study had never received ectoparasiticidal treatments and shared habitat with wildlife species and infected ticks. However, *H. felis* transmission routes are not known yet (Baneth et al., 2013) but ticks are suspectted vectors. Congenital transmission may also occur like for *H. canis* in dogs (Baneth et al., 2013; Murata et al., 1993). Predation was described for *H. americanum* in dogs feeding on rodents and rabbits infected with a quiescent cystozoite form of the parasite (Johnson et al., 2008; Johnson et al., 2009). Stray cats may engage more on predation than dogs and may ingest arthropod vectors when grooming. The time of the year when samples were collected would be an important factor conditioning prevalence. Tick abundance in Murcia peaks in late spring and in contrast to cats, no dogs were examined in the summer. Winter *Hepatozoon* spp. infections might be accounted for by transmission at this time of the year since *R. sanguineous* is active all year round (Estrada-Peña et al., 2017) and/or infection becoming chronic for a long time (Baneth et al., 1998). In Israel, *Hepatozoon* spp. was most prevalent in cats in winter (Baneth et al., 1998) and in dogs it was not seasonal (Baneth & Weigler, 1997). Prevalence of *Hepatozoon* spp. in dogs in Iran was greatest in summer (Barati & Razmi, 2018).

Compared to other studies in the Iberian Peninsula, the prevalence of *L. infantum* in cats here reported was slightly lower than the 26% to 30% found by Maia et al. (2008), Martín-Sánchez et al. (2007) and Millán et al. (2011), and higher than the 0% to 20% found in other studies (Alcover et al., 2021; Maia et al., 2010; Miró et al., 2014; Montoya et al., 2018; Sherry et al., 2011; Tabar et al., 2008). Prevalence estimations may differ depending on the number of cats sampled, the sample used for diagnosis and the animal's clinical status. Elevated parasitaemia is most typical in animals suffering clinical leishmaniosis whilst in asymptomatic dogs parasitaemia is less common than infection in skin and lymphoid tissue (Chitimia et al., 2011). Other studies comparing *L. infantum* prevalence in different cat samples report variable results. In stray and colony cats in Italy, PCR prevalence in blood was lower than in conjunctival swabs (Morganti et al., 2019) and marginally lower than in lymph node samples (Spada et al., 2020). Chatzis et al. (2014) reported similar PCR prevalence in blood and tissue in healthy cats and higher prevalence in tissues compared to blood in cats with skin lesions. Here, *L. infantum* infection was not associated to clinical signs typical of FL (Abramo et al., 2021; Pennisi et al., 2015). However, none of the clinical signs detected are specific of FeL and skin fitting wounds are common in stray cats.

High prevalence of *L. infantum* in winter is compatible with leishmaniosis' s long incubation period (Hernández et al., 2015; Oliva et al., 2006; Pennisi et al., 2012).

Our *L. infantum* and *Hepatozoon* spp. studies support a common domestic and sylvatic transmission cycle for these parasites in southeast Spain, and the need to considered cats as an element in leishmaniosis epidemiology. It also highlights the importance of controlling stray cats and abandoned dog populations to reduce parasite prevalence, their impact on pets and the risk of zoonotic transmission.

Conclusions

Hepatozoon and *L. infantum* are important parasites of stray cats and abandoned dogs in Mediterranean biotopes, and a better demographic and sanitary control of this vulnerable population is required. These parasites circulate in a transmission cycle common to domestic and wild animals. Cats have a potentially relevant role in the epidemiology of leishmaniosis. Tick distribution studies are required for a better understanding of *Hepatozoon* spp. and *Babesia* spp. in this part of Spain.

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CHAPTER 2

An epidemiological study in wild carnivores from Spanish Mediterranean ecosystems reveals association between *Leishmania infantum*, *Babesia* spp. and *Hepatozoon* spp. infection and new hosts for *Hepatozoon martis*, *Hepatozoon canis* and *Sarcocystis* spp.

Abstract

The aetiology and epidemiology of vector borne apicomplexan *Babesia* and *Hepatozoon* and kinetoplastid *Leishmania infantum* infections in wildlife have not been explored in wide areas of southern Spain. We investigated these infections in 151 wild carnivores, including foxes, badgers, beech martens, hedgehogs, wild cats, Egyptian mongooses, otters, genets and racoons. Overall, *Hepatozoon*, *Babesia* and *L. infantum* infections were detected in 68%, 48% and 23% of the wild animals surveyed, respectively. *L. infantum* infected wildlife were more likely to be also infected with the apicomplexan *Hepatozoon* and *Babesia* spp. compared to the non-infected counterparts ($p < .05$). We report for the first time *Hepatozoon martis* in badgers and wild cats and *H. canis* in beech martens, and a *Babesia* sp. in wild cats from Spain. Our results also indicate the widespread distribution of *H. canis* in foxes (91%) and beech martens (13%), *H. martis* in beech martens (81%), wild cats (20%) and badgers (13%), *H. felis* in wild cats (60%), *B. vulpes* in foxes (64%), *Babesia* sp. type A closely related to *B. vulpes*, in badgers (58%) and *Babesia* sp. in wild cats (20%). Moreover, *L. infantum* infection was found in foxes (29%), beech martens (13%), badgers (8%) and 1/3 Egyptian mongooses. We also detected *Cytauxzoon* sp. in a wild cat and the first *Sarcocystis* sp. in a genet. This study provided evidence of increased risk of *L. infantum* infection in wildlife animals co-infected with *Babesia* spp. or *Hepatozoon* spp. and indicated that these infections are widespread in wild carnivores from Spanish Mediterranean ecosystems.

Introduction

Vector-borne infections (VBI) are an emerging problem worldwide, associated to factors influencing vector distribution such as climate warming and increased host exposure to vectors associated to modern lifestyle. Most VBI are transmitted by ticks and insects, and affect humans and animals in interconnected domestic and sylvatic transmission cycles (Ortuño et al., 2019; Tomassone et al., 2018). *Babesia* and *Hepatozoon* spp. are widespread apicomplexan protozoans transmitted by ticks during their feeding process in the case of *Babesia* spp. and by ingestion of infected arthropod vectors in the case of *Hepatozoon* spp. Parasites of these two genera show a variable degree of host specificity. *Babesia* and *Hepatozoon* species infecting carnivores are characterized by molecular methods and

display morphological differences in some cases. *Babesia* spp. infection may cause severe haemolytic syndromes but latent infections are also possible, and their virulence varies between species and the susceptibility of their different hosts (Irwin, 2010; Solano-Gallego & Baneth, 2011). Infection with *Hepatozoon* spp. is sometimes mild or asymptomatic, but severe illness has been reported (Baneth, 2011; Baneth et al., 2007). On the other hand, *Leishmania* is a kinetoplastid protozoan parasite infecting macrophages and monocytes, transmitted by sand flies whose vector transmission is restricted to the warm latitudes of the planet including Spain, where *Leishmania infantum* is the only endemic *Leishmania* species. The dog is the primary domestic reservoir of this parasite, but infection has been described in a wide range of host species, including humans. Whilst most *Leishmania*-infected animals remain asymptomatic, infection may be life threatening particularly to dogs and people (Solano-Gallego & Baneth, 2008).

Babesia, *Hepatozoon* and *Leishmania* coinfections have been reported and are to be expected where ticks, sand fly vectors and their hosts share a habitat (Medkour, Laidoudi, Lafri, et al., 2020; Medkour, Laidoudi, Marié, et al., 2020; Yisaschar-Mekuzas et al., 2013). Yet, few studies have investigated the clinical and immunopathological mechanisms of coinfections with these parasites (De Tommasi et al., 2014), and the risk of coinfections has rarely been studied to evaluate the epidemiological relationships between them (Mekuzas et al., 2009; Toepp et al., 2019). Th1 cell-mediated immune responses are considered the most effective line of defence against intracellular parasites (Day, 2011). Indeed, animals with a limited capacity to elicit a cellular response are more susceptible to develop clinical leishmaniosis, and it is influenced by inheritable traits (Iborra et al., 2007; Solano-Gallego et al., 2000). Coinfections may also be facilitated by immunosuppression resulting from these parasites targeting immune response mechanisms and modifying them.

The present study investigated *Babesia*, *Hepatozoon* and *Leishmania* infections in wild carnivores from southern Spain. This is a typical *L. infantum* endemic area and infection with this parasite has been reported in humans, dogs and wildlife (Chitimia et al., 2011; Ortuño et al., 2017; Risueño et al., 2018; Sobrino et al., 2008), but information on infection with haemoprotozoa apicomplexan parasites in wildlife from this area is scarce. Therefore, the objectives of this study were to estimate the spatial and temporal distribution of infection with these parasites, characterise the piroplasmid and *Hepatozoon* spp. found by DNA sequencing and investigate the risk of coinfection between these and *L. infantum*.

Materials and methods

Study population and experimental design

The study included samples from 151 wild animals from the autonomous communities of Murcia and Valencia in southeast Spain, Andalusia in the south and Extremadura in the southwest (Figure 1), collected between 2011 and 2018. It included 89 red foxes (*Vulpes vulpes*), 24 badgers (*Meles meles*), 16 beech martens (*Martes foina*), 6 hedgehogs (*Erinaceus europaeus*), 5 wild cats (*Felis silvestris)*, 3 Egyptian mongooses (*Herpestes ichneumon*), 3 genets (*Genetta genetta*), 3 otters (*Lutra lutra*) and 2 raccoons (*Procyon lotor*). Except for racoons which are introduced and are an invasive species, the rest of the species are native to Spain including genets and Egyptian mongooses which were introduced centuries ago and considered naturalized. Animals were either found dead by local authorities, legally hunted (foxes) or had died whilst being cared for at wildlife rescue centres following injury.

Figure 1. Areas in southern Spain where the study was performed.

All 151 animals were tested for *Babesia* spp. and *Hepatozoon* spp. using DNA extracted from spleen samples, which provides a high diagnostic sensitivity to detect infection by these parasites (Cardoso et al., 2014). In contrast, *L. infantum* diagnosis in this study was performed for 75 animals from south and southwest Spain (31 foxes, 20 badgers, 7 beech martens, 2 wild cats, 6 hedgehogs, 3 Egyptian mongooses, 3 genets and 3 otters), and the remaining 76 animals included had already been analysed for *Leishmania* infection (Risueño et al., 2018). *L. infantum* tests results from all 151 animals were used in the present study to evaluate risk factors and coinfection with other parasites. *L. infantum* diagnoses were performed using DNA from spleen samples, all of which provide a high diagnostic sensitivity for detecting *L. infantum* infection (Chitimia et al., 2011; de Andrade et al., 2006). Data from animals included the following demographic variables: sex, age, date, and place of capture.

DNA extraction

DNA was extracted using an automatized system (Maxwell® Promega, Madison, WI, USA) or a commercial kit ("HigherPurity™ Tissue DNA Purification Kit", Canvax©, Spain). DNA concentration and purity were analysed using the NanoDrop 2000® spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA).

Screening for *Babesia* spp. and *Hepatozoon* spp. by generic PCR amplification

Conventional PCR was performed to amplify an approximately 360 base pair (bp) of the common *Babesia* spp. and *Hepatozoon* spp. 18S ribosomal RNA sequence (rRNA) using primers Piroplasmid F (5′-CCA GCA GCC GCG GTA ATT C-3′) and Piroplasmid R (5′-CTT TCG CAG TAG TTY GTC TTT AAC AAA TCT-3′) (Tabar et al., 2008). PCR was performed using the Syntezza PCR-Ready High Specificity kit (Syntezza Bioscience, Israel), and 1 µl of each primer (10 µM), 19 µl Ultra-Pure Water (UPW) and 4 µl DNA. The following conditions were used for amplification: 95°C for 5 min; 35 cycles of 95°C for 30 s, 64°C for 45 s, and 72°C for 30 s; and 72°C for 5 min (Margalit Levi et al., 2018).

PCR assays included negative and positive DNA controls from a laboratory-bred pathogen-free dog and from dogs naturally infected with *Babesia vogeli* or *Hepatozoon canis*, and non-template DNA controls (NTC). PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and evaluated under UV

light for the size of amplified fragments by comparison to a 100 bp DNA molecular weight marker.

Specific *Babesia* spp. and *Hepatozoon* spp. PCR amplifications

An additional PCR using specific primers for *Babesia* spp. or *Hepatozoon* spp. 18S ribosomal RNA gene was performed in samples testing positive to the Piroplasmid PCR. The *Babesia* species-specific PCR amplified a 408-bp 18S RNA sequence with PIRO-A (5′-AAT ACC CAA TCC TGA CAC AGG G-3′) and PIRO-B (5′-TTA AAT ACG AAT GCC CCC AAC -3′) primers (Olmeda et al., 1997). The PCR was run with the same water, DNA and primers concentrations as described above and the conditions used for amplification were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 72°C for 5 min (Barbosa et al., 2020).

The *Hepatozoon* species-specific PCR amplified a 574-bp 18S RNA sequence with the *Hepatozoon* 18S forward (5´- GGT AAT TCT AGA GCT AAT ACA TGA GC -3´) and reverse (5´- ACA ATA AAG TAA AAA ACA YTT CAA AG -3´) primers (Almeida et al., 2012). The PCR was run with the same water, DNA and primers concentrations as described above and the conditions used for amplification were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and 72°C for 5 min (Sarma et al., 2019).

DNA sequencing

All positive PCR products were sequenced at the Center for Genomic Technologies, Hebrew University of Jerusalem with both forward and reverse primers.

The sequences were aligned and analysed using the ClustalW alignment program (Larkin et al., 2007) and the MEGA X software (Kumar et al., 2018), respectively, and compared to sequences deposited in GenBank, using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). A result was considered positive for a certain pathogen if it was the first match on BLAST and had at least a 97% identity with a known GenBank accession.

Leishmania infantum detection

Samples from animals analysed by Risueño et al. (2018) had been previously tested using the TaqMan probe real-time PCR (rt-PCR) assay for kinetoplast DNA (kDNA) described by Mary et al., (2004). Samples analysed for the present study were tested using

a *Leishmania*-FAST15 TaqMan probe rt-PCR assay similarly targeting kDNA sequences, with primers LEISH-1 (5'-AACTTTTCTGGTCCTCCGGGTAG-3') and LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3'), and TaqMan-MGB probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB) (Dantas-Torres et al., 2017; Francino et al., 2006). The *L. infantum* parasite load was estimated by considering the rt-PCR amplification threshold cycle (CT) at which a near logarithmic PCR product generation is detected (Gomes et al., 2008). Samples with a Ct>38 were considered negative.

Phylogenetic and genetic distance analyses

Phylogenetic and genetic distance analyses were performed using the Molecular Evolutionary Genetics Analysis software MEGAX (Kumar et al., 2018). The Maximum-Likelihood method was used to infer tree topology. Confidence values for individual branches of the resulting trees were determined by bootstrapping analysis considering a 70% or higher value significant. Homologous sequence of *Plasmodium falciparum* (accession number MF155937) was used as outgroup sequences for *Babesia, Cytauxzoon* and *Sarcocystis* spp. phylograms, while *Sarcocystis cymruensis* (MG564723) was used for the *Hepatozoon* spp. trees.

Genetic distance (expressed in %) among a selection of representative sequences was calculated using the Kimura 2-parameter substitution model with gamma distributed (G) rates and pairwise deletion to treat gaps and/or missing data (Kimura, 1980), implemented in the MEGA X software.

Statistical analysis

The proportion of infected foxes and badgers (the most abundant species tested) and median *L. infantum* PCR CTs among positive animals, were compared according to levels of categorised explanatory variables using Fisher's exact test and the non-parametric Kruskal-Wallis test, respectively. Explanatory variables were age (young or adult), collection years (two equal size categories: 2013-15 and 2016-18 for foxes, and 2011-14 and 2015-17 for badgers), season and geographical origin of the animals (southeast and south-southwest). Statistical significance was taken for $p < 0.05$ for a two-tailed test. All analyses were performed with R software (http://cran.r-project.org/).

Results

Frequency of *Babesia* spp., *Hepatozoon* spp. and *L. infantum* infection

Among the 151 animals analysed, 123 (81%) were positive to at least one pathogen. The overall infection prevalence (range in positive host species) was 48% (20-64%) for *Babesia* spp., 68% (13-94%) for *Hepatozoon* spp. and 21% (8-33%) for *L. infantum* (Table 1). *Leishmania infantum* prevalence in samples from the south and southwest of Spain tested here for the first time was 5% (3-33%). Median (range) PCR CTs among *L. infantum* positive animals were 34 (10-38) for all host species, and 34 (10-38), 37 (37-38), 38 (37-38) and 38 (38-38) for foxes, badgers, beech martens and the Egyptian mongoose, respectively ($p<0.05$).

In foxes, the prevalence of *B. vulpes*, *H. canis* and *L. infantum* were higher in the southeast compared to south and southwest of Spain (78% vs. 39%, 100% vs. 74% and 46% vs. 3%, respectively) ($p<0.05$) and in 2013-2015 compared to 2016-2018 (80% vs. 42%, 98% vs. 79% and 52% vs. 12%, respectively) ($p<0.05$). Similarly, in badgers, *H. martis* prevalence was higher in the southeast compared to the south and southwest (75% vs. 0%) ($p<0.05$). None of the other associations between infection and explanatory variables were significant ($p>0.05$).

In addition, PCR amplification with the piroplasmid primers allowed identifying *Sarcocystis* spp. infection in one of three genets and *Cytauxzoon* sp. in one of five wild cats.

Table 1. Number (%) of Babesia spp., Hepatozoon spp. and Leishmania infantum PCR-positive wildlife animal species detected from spleen samples.

Wild species	**N**	***Babesia***			***Hepatozoon***				***L. infantum*[1]**	**At least one pathogen**
		vulpes	**other *Babesia* spp.**	**all**	***canis***	***felis***	***martis***	**all**		
Foxes	89	57 (64)	0 (0)	**57 (64)**	81 (91)	0 (0)	0 (0)	**81 (91)**	**26 (29)**	**87 (98)**
Badgers	24	0 (0)	14 (58)	**14 (58)**	0 (0)	0 (0)	3 (13)	**3 (13)**	**2 (8)**	**15 (63)**
Beech martens	16	0 (0)	0 (0)	**0 (0)**	2 (13)	0 (0)	13 (81)	**15 (94)**	**2 (13)**	**15 (94)**
Wild cats	5	0 (0)	1 (20)	**1 (20)**	0 (0)	3 (60)	1 (20)	**4 (80)**	**0 (0)**	**5 (100)**
Hedgehogs	6	0 (0)	0 (0)	**0 (0)**	0 (0)	0 (0)	0 (0)	**0 (0)**	**0 (0)**	**0 (0)**
Egyptian mongooses	3	0 (0)	0 (0)	**0 (0)**	0 (0)	0 (0)	0 (0)	**0 (0)**	**1 (33)**	**1 (33)**
Otters	3	0 (0)	0 (0)	**0 (0)**	0 (0)	0 (0)	0 (0)	**0 (0)**	**0 (0)**	**0 (0)**
Genets	3	0 (0)	0 (0)	**0 (0)**	0 (0)	0 (0)	0 (0)	**0 (0)**	**0 (0)**	**0 (0)**
Raccoons	2	0 (0)	0 (0)	**0 (0)**	0 (0)	0 (0)	0 (0)	**0 (0)**	**0 (0)**	**0 (0)**
All	151	57 (38)	15 (10)	**72 (48*)**	83 (55)	3 (2)	17 (11)	**103 (68*)**	**31 (21)**	**123 (81)**

* Significant differences between species ($p<0.001$)

[1] Seventy-six of these animals were previously analysed for *L. infantum* in the study of Risueño et al. (2018).

DNA sequence characterization of *Babesia* spp., *Hepatozoon* spp., *Cytauxzoon* spp. and *Sarcocystis* spp., and phylogenetic and genetic distance analyses

The 57 fox *Babesia* spp. sequences (isolates BV1-3) corresponded to *B. vulpes*, with a 99.7-100% identity with *B. vulpes* from a red fox from Iraq (MK957183) and *B. vulpes* from a dog from Galicia in NW Spain (MK585200) (Table 2). The fox *B. vulpes* BV1-3 sequences from this study clustered together in a phylogenetic tree with homologous GenBank *B. vulpes* sequences from dogs and foxes and were separated from sequences of other close *Babesia* spp. with a bootstrap value of 93% (Figure 2). *Babesia* spp. sequences from 14 badgers (BSP1 and BSP3) were 100% similar to *Babesia* sp. badger type A from badgers in China (MG799845) and the United Kingdom (MT077002). In the phylogenetic analysis, *Babesia* sp. types A and B from badgers clustered separately (98% and 99% bootstrap values, Figure 2) and were close to *B. vulpes* sequences (0.9-5.5% nucleotide divergence, Table 1S). The wild cat *Babesia* sequence (BSP2) had a 98.7% similarity to *Babesia* sp. from foxes in Israel and golden jackals from Iraq (KJ956782 and MK957184), clustering together (91% bootstrap value), and separated to *Babesia* sp. in wild carnivores from Africa but closely related to them (4.1-5.3% nucleotide divergence; Figure 2, Table 1S).

Table 2. Results of the BLAST analysis for the Babesia spp. and Hepatozoon spp. positive samples.

New GenBank accession No.	Isolate	Parasite species	Wildlife mammalian host (N)	Primers	Seq. length (bp)	Closest GenBank accession No.	Identity (%)	Query cover (%)
MW578971	BSP1	*Babesia* sp. badger type A	Badger (8)	Piropl-F/R	311	MT077002	100	100
MW578972	BSP2	*Babesia* sp.	Wild cat (1)	Piropl-F/R	312	MK957184	98.7	100
MW578973	BSP3	*Babesia* sp. badger type A	Badger (6)	Piro-A/B	370	MG799845	100	100
MW578974	BV1	*Babesia vulpes*	Fox (4)	Piropl-F/R	316	MK957183	100	100
MW578975	BV2	*Babesia vulpes*	Fox (51)	Piro-A/B	375	MK585200	100	100
MW578976	BV3	*Babesia vulpes*	Fox (2)	Piro-A/B	375	MK585200	99.7	100
MW578979	HC1	*Hepatozoon canis*	Fox (18)	Piropl-F/R	337	MN628317	100	100
MW578980	HC2	*Hepatozoon canis*	Fox (2)	Piropl-F/R	337	MN628318	100	100
MW578981	HC3	*Hepatozoon canis*	Beech marten (1)	Piropl-F/R	337	MN628318	100	100
MW578982	HC4	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MN791089	99.7	100
MW578983	HC5	*Hepatozoon canis*	Fox (44)	Piropl-F/R	337	MN791089	100	100
MW578984	HC6	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MN628317	99.1	100
MW578985	HC7	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MK645967	99.1	100
MW578986	HC8	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MN628317	99.4	100
MW578987	HC9	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MN628317	99.7	100
MW578988	HC10	*Hepatozoon canis*	Fox (4)	Piropl-F/R	337	MN628329	99.7	100
MW578989	HC11	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MK645967	100	100
MW578990	HC12	*Hepatozoon canis*	Fox (1)	Piropl-F/R	337	MK645967	99.4	100
MW578991	HC13	*Hepatozoon canis*	Fox (3)	Piropl-F/R	337	KP216425	100	100
MW578992	HC14	*Hepatozoon canis*	Beech marten (1)	Hep18S-F/R	515	MK957188	99.6	100
MW578993	HC15	*Hepatozoon canis*	Fox (3)	Hep18S-F/R	515	MK757802	99.8	100
MW578994	HF1	*Hepatozoon felis*	Wild cat (1)	Piropl-F/R	337	MG386483	100	100
MW578995	HF2	*Hepatozoon felis*	Wild cat (1)	Piropl-F/R	337	MG386484	99.1	100
MW578996	HF3	*Hepatozoon felis*	Wild cat (1)	Hep18S-F/R	515	AY628681	100	100
MW578997	HM1	*Hepatozoon martis*	Wild cat (1)	Piropl-F/R	337	MG136688	99.4	100
MW578998	HM2	*Hepatozoon martis*	Beech marten (2)	Piropl-F/R	337	MG136688	99.4	100
MW578999	HM3	*Hepatozoon martis*	Beech marten (10)	Piropl-F/R	337	MG136688	100	100
MW579000	HM4	*Hepatozoon martis*	Badger (1)	Piropl-F/R	337	MG136688	100	100
MW579001	HM5	*Hepatozoon martis*	Badger (2)	Hep18S-F/R	515	MH656728	100	100
MW579002	HM6	*Hepatozoon martis*	Beech marten (1)	Hep18S-F/R	515	MH656728	100	100
MW578977	CYT1	*Cytauxzoon* sp.	Wild cat (1)	Piropl-F/R	307	MT904044	100	100
MW578978	SC1	*Sarcocystis* sp.	Genet (1)	Piropl-F/R	325	MG564723	100	100

Figure 2. Phylogenetic tree of Babesia spp. sequences from wild carnivores in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 236 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

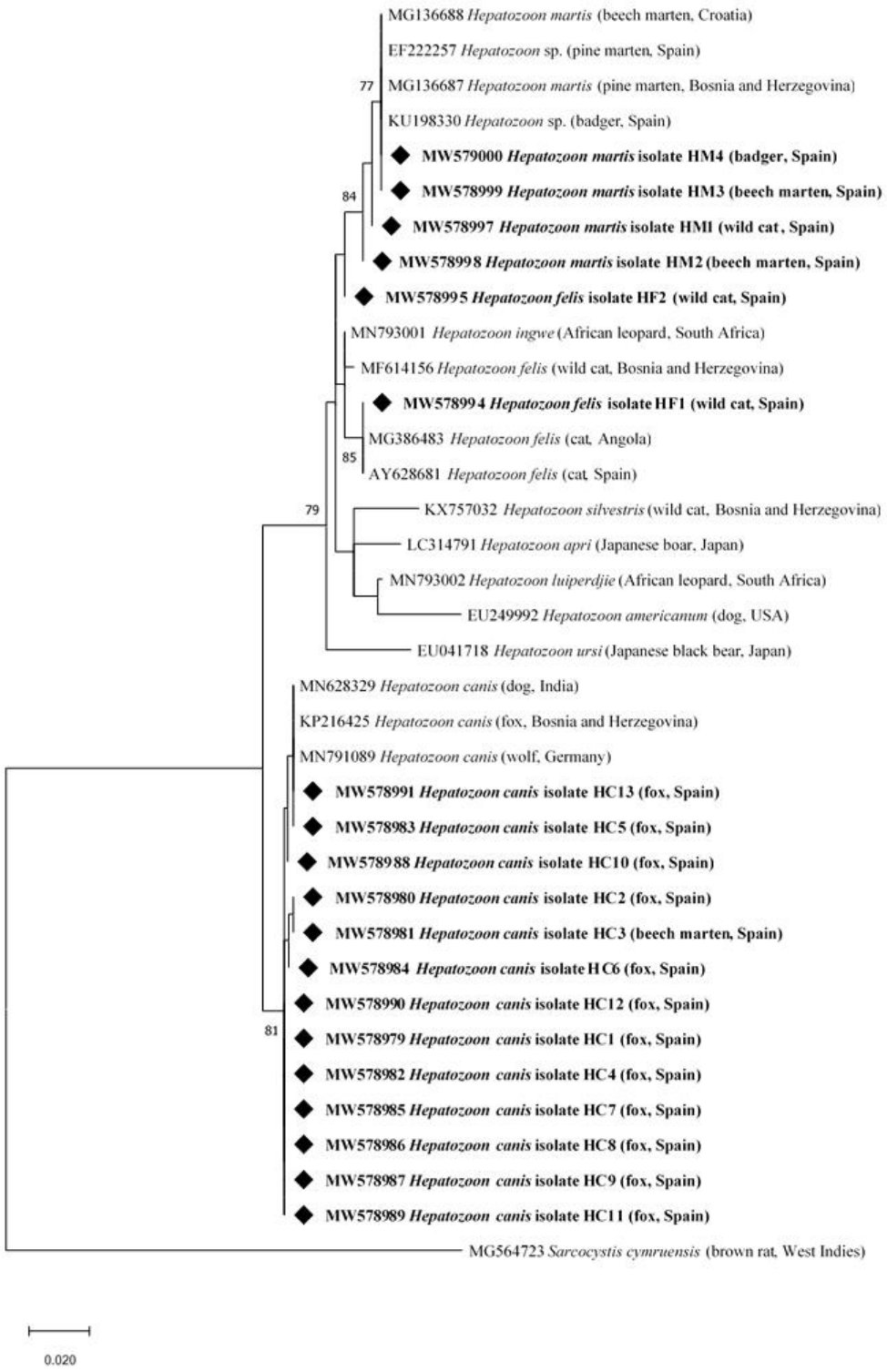

Figure 3. Phylogenetic tree of Hepatozoon spp. sequences with Piroplasmid F/R primers from wild carnivores in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 332 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Figure 4. Phylogenetic tree of Hepatozoon spp. sequences with Hep18S F/R primers from wild carnivores in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Hepatozoon spp. from all foxes (isolates HC1-2, HC4-13 and HC15) and two beech martens (HC3 and HC14) were 99.1-100% identical to *H. canis* (MN628317, MN628318, MK645967, MN791089, MN628329, KP216425, MK957188 and MK757802) (Table 2). The *Hepatozoon* spp. samples from the remaining 13 beech martens (HM2-3 and HM-6) and from 3 badgers (HM4 and HM5) and 1 wild cat (HM1) were 99.4-100% identical to *H. martis* (MG136688 and MH656728). The *Hepatozoon* sp. from the three other wild cats (HF1-3) had a 99.1-100% identity with *H. felis* (MG386483, MG386484 and AY628681) (Table 2). In the phylogenetic trees, *H. canis* sequences from foxes and beech martens clustered together with homologous sequences found in canids, presenting less than 3% of nucleotide divergence (Table 2S), and were found to segregate with a 81-96% bootstrap into a different clade from other *Hepatozoon* spp. (Figures 3 and 4). *H. felis* sequences from wild cats grouped together with homologous sequences from domestic and wild felids (bootstrap value 85-99%; Figures 3 and 4) and presented less than 1% nucleotide divergence (Table 3S). The *H. martis* sequences retrieved from badgers, wild cats and beech martens presented less than 2% nucleotide divergence (Table 3S), and clustered together in a separate clade from *H. felis* sequences with a bootstrap value of 76-84% (Figures 3 and 4).

The *Cytauxzoon* sp. sequence found in a wild cat was 100% identical to a common fragment in three new *Cytauxzoon* species (*C. europaeus*, *C. otrantorum* and *C. banethi*) described in wild felids from European countries, named by Panait et al. (2021) (MT904044, MT904033, MT904029) (Table 2). They all clustered together (99% bootstrap value; Figure 5) and were different from *C. felis* (3.1-8.2% of nucleotide divergence; Table 4S).

A *Sarcocystis* sp. sequence found in a genet was 100% identical to *S. cymruensis* from brown rats from China and Grenada (West Indies) and to *S. muris* from a cat from Egypt, and 99.1% identical to *S. muris* from mice (MT372786, MG564723, KC877996, M64244). Consequently, it clustered together with *Sarcocystis* spp. sequences from these rodents (100% bootstrap value), and separately to those retrieved from wild carnivores (Figure 6). The estimated evolutionary divergence for the *Sarcocystis* sp. sequence from the genet was 0% when compared to *S. cymruensis* from brown rats, *S. muris* from a cat, and *S. rodentifelis* from a vole, and 0.6% compared to *S. muris* (M64244) from a mouse (Table 5S).

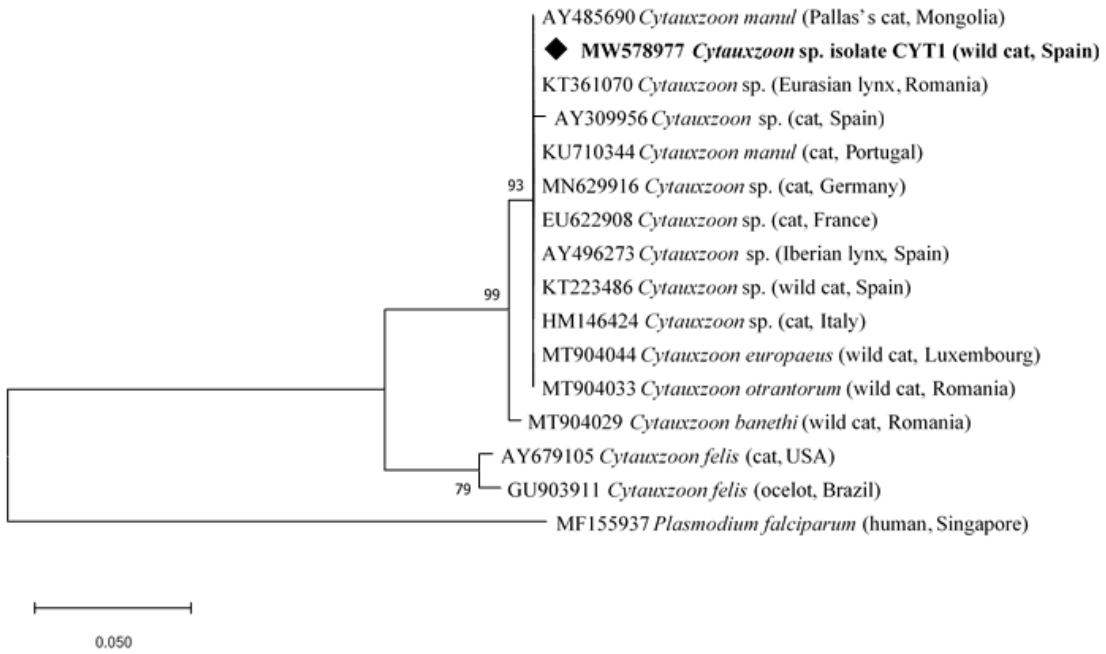

Figure 5. Phylogenetic tree of Cytauxzoon spp. sequence from a wild cat in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 260 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

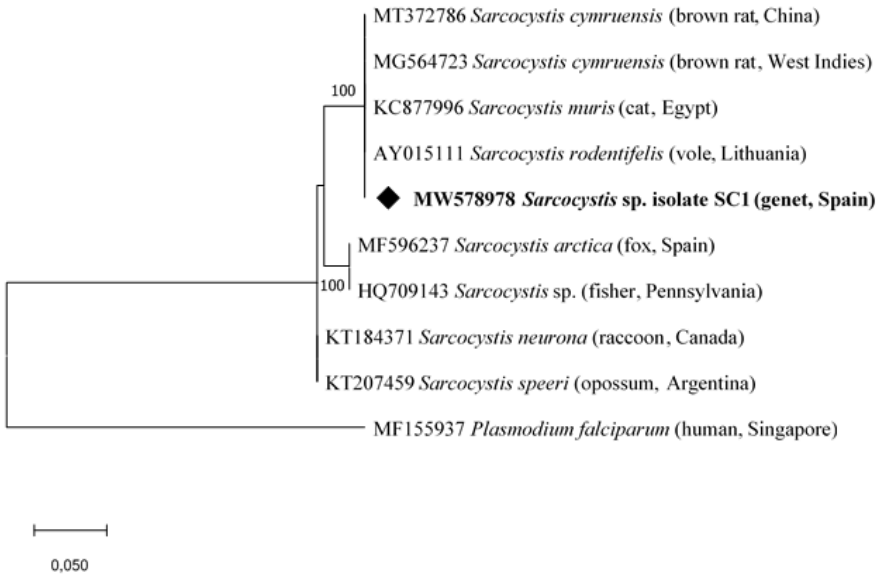

Figure 6. Phylogenetic tree of Sarcocystis spp. sequence from a genet in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 323 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Coinfections

The prevalence of *Babesia* spp. or *Hepatozoon* spp. was significantly higher in *L. infantum*-positive wild animals compared to *L. infantum*-negative individuals ($p<0.05$, Table 3). When considering individual host species, this relationship was marginally significant only in foxes between *L. infantum* and *B. vulpes* or *H. canis* ($p<0.10$) (Table 6S).

Table 3. Relationship between Babesia, Hepatozoon and L. infantum infections.

		***Babesia* spp.**			***Hepatozoon* spp.**		***L. infantum*[1]**	
Parasite	**Infected**	**No.**	**% +ve.**	***P* value**	**% +ve.**	***P* value**	**% +ve.**	***P* value**
Babesia spp.	Yes	72	-	-	74	0.2209	32*	0.0011
	No	79	-		63		10	
Hepatozoon spp.	Yes	103	51	0.2209	-	-	27*	0.0024
	No	48	40		-		6	
L. infantum[1]	Yes	31	74*	0.0011	90*	0.0024	-	-
	No	120	41		63		-	

[1] Data of *L. infantum* infection in wild carnivores from southwest (N=75) and southeast (N=76) Spain. The *L. infantum* results from southeast Spain were part of a previous study (Risueño et al., 2018) and included here because they were analysed for additional pathogens in the present study.

Thirty foxes were coinfected with *H. canis* and *B. vulpes*, 5 foxes with *H. canis* and *L. infantum* and 21 foxes were coinfected with all three pathogens. Two badgers had *Babesia* sp. type A and *H. martis*, and another two had *Babesia* sp. type A and *L. infantum*. One beech marten was infected with *H. canis* and *L. infantum* and another one had *H. martis* and *L. infantum* infections. Finally, one wild cat was coinfected with *H. felis* and *Cytauxzoon* sp. (Table 4).

Table 4. *Babesia* spp., *Hepatozoon* spp. and *L. infantum* coinfections in different wildlife hosts. The number (%) of animals positive/negative to each coinfection are included.

Parasites	**Fox**	**Badger**	**Beech marten**	**Wild cat**	**Genet**	**Egyptian mongoose**	**Hedgehog**	**Otter**	**Raccoon**	**All**
	N=89	**N=24**	**N=16**	**N=5**	**N=3**	**N=3**	**N=6**	**N=3**	**N=2**	**N=151**
Double infections										
B. vulpes + *H. canis*	30 (34)	0	0	0	0	0	0	0	0	30 (20)
Babesia sp. type A + *H. martis*	0	2 (8)	0	0	0	0	0	0	0	2 (1)
Babesia sp. type A + *L. infantum*	0	2 (8)	0	0	0	0	0	0	0	2 (1)
H. canis + *L. infantum*	5 (6)	0	1 (6)	0	0	0	0	0	0	6 (4)
H. martis + *L. infantum*	0	0	1 (6)	0	0	0	0	0	0	1 (1)
H. felis + *Cytauxzoon* sp.	0	0	0	1 (20)	0	0	0	0	0	1 (1)
Triple infections										
B. vulpes + *H. canis* + *L. infantum*	21 (24)	0	0	0	0	0	0	0	0	21 (14)

Discussion

This study describes for the first time the prevalence and characterization of *Babesia* spp., *Hepatozoon* spp. and *L. infantum* in wildlife in previously uncharted areas of southern Spain and provides an account of the risk of coinfections between these parasites. *Hepatozoon* spp. and piroplasmid spp. infections were widespread among wildlife. *L. infantum* prevalence was comparatively lower, and those animals infected were also more likely to have *Babesia* spp. and *Hepatozoon* spp. compared to those not infected with the apicomplexans. Furthermore, it is the first report of *H. canis* infection in a beech marten and of *H. martis* in wild cats. We also detected *H. martis* in three badgers, which agrees with the finding of Barandika et al. (2016), who reported infection with a *Hepatozoon* sp. in a badger from northern Spain which, due to the sequence identity, was probably the species described later as *H. martis* in martens by Hodžić et al. (2018). *Hepatozoon* spp. infection has been scarcely and only locally investigated in wildlife in Spain. The overall 68% prevalence of infection found in the present study suggests that these parasites are very common and efficiently transmitted in sylvatic environments. Moreover, based on the present study and other reports, *Hepatozoon* spp. seem to have low host specificity (Criado-Fornelio et al., 2006, 2007; Smith, 1996). However, there were significant differences in the prevalence of *Hepatozoon* spp. infection between host species, ranging from 80-91% in foxes, wild cats and beech martens, 13% in badgers and 0% in all other species. Similarly, high prevalences were detected in foxes in the Iberian Peninsula, including 76% in Portugal and 90% in Guadalajara, central Spain (Cardoso et al., 2014; Criado-Fornelio et al., 2006). Likewise, *Hepatozoon* spp. infection in badgers was less common in previous studies, and estimated prevalence values were 1% in northern Spain (Barandika et al., 2016) and 0% in central Spain and northern Italy (Battisti et al., 2020; Gimenez et al., 2009). The reasons for such diverging differences between host species could be related to the epidemiology of each *Hepatozoon* sp., with relation to the abundance of its competent vectors in different areas, other possible modes of transmission such as transmission by carnivorism or transplacentally, and the abundance of other sympatric species infected with the same species in the study region. In addition to ticks, certain *Hepatozoon* spp. are transmitted by other arthropods (Smith, 1996) as well as transplacentally (Murata et al., 1993) and by carnivorism (Smith, 1996; Johnson et al., 2009). However, the relative importance of these modes of transmission is unknown. Moreover, no transmission route

has yet been described for *H. felis* and *H. martis* (Baneth et al., 2013; Hodžić et al., 2018). *H. canis* is efficiently transmitted by *Rhipicephalus sanguineus*, which is the most prevalent and abundant tick species in dogs in urban and rural areas in Spain (Checa et al., 2019; Estrada-Peña et al., 2017), but much less so in foxes in Spain (<4% prevalence and <1% abundance) (Checa et al., 2018; Sanchis Monsonís, 2016; Serrano Barrón, 2004), except in Murcia, where 55% of foxes were shown to be infested with *R. sanguineus* (Martínez-Carrasco et al., 2007).

The high prevalence (64%) of *B. vulpes* in foxes is in agreement with other studies from the Atlantic northwest of the Iberian Peninsula (63-100%) and central Spain (50%) (Cardoso et al., 2013; Criado-Fornelio et al., 2007; García, 2006; Miró et al., 2015). Our results indicate that this parasite is also efficiently transmitted in southern Spain. Animals may become infected with *Babesia* spp. by tick vectors, congenitally and possibly by blood exchange through dog fighting (Yeagley et al., 2009). *Ixodes hexagonus* was considered a potential vector of *B. vulpes* in northwest Spain based on its abundance and the detection of DNA of the parasite in this tick species (Camacho et al., 2003; Checa et al., 2018). However, *I. hexagonus* was not detected in a previous study in Murcia (Martínez-Carrasco et al., 2007) or in other Southern and Mediterranean regions in Spain (Estrada-Peña et al., 2017), except in the Autonomous Community of Valencia where it was found in 20% of 272 foxes analysed (Sanchis, 2016). *B. vulpes* is also prevalent in areas where *I. hexagonus* is not a common tick including the United States (Birkenheuer et al., 2010). Clearly, other transmission modes exist for *B. vulpes* that need to be investigated (Barash et al., 2019; Simões et al., 2011).

Babesia spp. infection was also widespread in badgers and it was detected in one wild cat for the first time in Spain. Prevalence in badgers (58%) was higher than in a previous study in northern Spain, where 35% (43/122) of badgers were *Babesia* sp. positive (Barandika et al., 2016). Barandika et al. (2016) described two variants of badger-associated *Babesia* sp., named “type A” and “type B” which have been reported elsewhere in Europe and China (Barandika et al., 2016; Bartley et al., 2017; Guardone et al., 2020; Hornok et al., 2018; Santoro et al., 2019). *Babesia* sp. type A found here in badgers is closely related to *B. vulpes*. In contrast, the sequence from the wild cat is close to *Babesia* sp. from golden jackals from Iraq and red foxes from Israel, and more distantly related to *B. lengau* from cheetahs and domestic cats in South Africa (Bosman et al., 2010; Margalit Levi et al., 2018; Otranto et al., 2019). The low prevalence of *Babesia* spp. in wild cats and

its absence in stone martens, genets, otters, hedgehogs, Egyptian mongooses and raccoons in the present study is in agreement with other surveys in some of these wildlife species in Europe (Millán et al., 2016; Santoro et al., 2019). However, the number of animals belonging to some of these species that we tested was small.

The high apicomplexan infection prevalence in wildlife described here, along with common contact between wildlife and domestic animals in periurban environment (Mackenstedt et al., 2015), highlights the potential risk of infection spill-over from sylvatic to domestic environments which may have major health consequences. Examples of this include *B. vulpes* causing severe infection in dogs in Portugal and Spain (Camacho et al., 2004), *H. americanum* in coyotes severely affecting dogs in the USA (Panciera & Ewing, 2003) and *C. felis* from bobcats causing serious illness in domestic cats (Birkenheuer et al., 2006).

We also report the detection of *Cytauxzoon* sp. in a wild cat and *Sarcocystis* spp. in a genet. The wild cat came from Jaén province (Andalusia), where Iberian lynxes are also infected with *Cytauxzoon* spp. (García-Bocanegra et al., 2010; Meli et al., 2009; Millán et al., 2007), and this parasite is considered a potential threat to this endangered feline species (Millán et al., 2009). *Cytauxzoon* spp. has been also been reported in domestic (0.8-23%) and wild cats (67-79%) in Spain and other European countries (Barandika et al., 2016; Carli et al., 2012; Criado-Fornelio et al., 2009; Díaz-Regañón et al., 2017; León et al., 2017). However, *Cytauxzoon* sp. infection in Europe is less pathogenic than *C. felis* in the USA, transmitted by *Amblyomma americanum*, a tick species from North America not present in Europe (Birkenheuer et al., 2006; Reichard et al., 2010). It was recently reported that there are at least three different species of *Cytauxzoon* which infect wild felids in Europe and are genetically distinct (Panait et al., 2021). *Sarcocystis* spp. have not been previously reported in genets and the sequence found in this study was identical to *S. cymruensis* from brown rats from China and the West Indies, and to *S. muris* from a cat from Egypt (Al-Kappany et al., 2013; Murata et al., 2018). *Sarcocystis* spp. have an indirect host-prey life cycle. The definite host of *S. cymruensis* has not been identified yet and could be genets, as they feed on rodents (Amroun et al., 2014; Sánchez et al., 2008). This hypothesis would need to be confirmed by detection of intestinal sexual stages and presence of oocysts or sporocysts in the genet's faeces. *S. cymruensis* in this study was found in a spleen sample, which is a possible location for extra-intestinal *Sarcocystis* spp. stages which are increasingly reported in muscle tissue from carnivore definitive hosts (Máca, 2018; Prakas et al., 2019).

L. infantum infection prevalence in southern and southwestern Spain (5%) was lower than in other PCR surveys in wild carnivores in this part of Spain, specifically in Huelva (15%) (Sobrino et al., 2008) and Extremadura (75%) (Muñoz-Madrid et al., 2013). Differences in *L. infantum* prevalence would be associated to variable sandfly infection rates and distribution, which is related to macro- and micro-environmental factors, and the presence of other susceptible hosts (Muñoz et al., 2017; Muñoz et al., 2019; Risueño et al., 2017). Canine leishmaniosis is widespread in southern Spain but seroprevalence is similarly variable depending on the area and population examined (Gálvez et al., 2020).

The high prevalence of *L. infantum*, *Babesia* spp. and *Hepatozoon* spp. in the southeast of Spain compared to other regions in this study, indicates a greater infection pressure in this region to the three parasites and possibly, synergism between them. Coinfections with these parasites have been described before (Miró et al., 2015; Tabar et al., 2009), but the immunopathological mechanisms facilitating *Leishmania*, *Babesia* and *Hepatozoon* spp. coinfection remain unknown. Previous studies suggested synergism between *L. infantum* and *Ehrlichia canis*, due to common target cells and overlapping immunopathologic pathways, and *Ehrlichia* spp., *Anaplasma* spp. and other VBI increased the risk of *Leishmania* spp. seropositivity and clinical disease in dogs from the USA and Brazil (Attipa et al., 2018; Toepp et al., 2019). Therefore, wildlife mammals infected with *L. infantum* in southern Spain are likely to be infected also with other VBI such as *Babesia* and *Hepatozoon* spp. and should be tested and potentially treated for co-infection.

Conclusions

This study indicated that *Hepatozoon* spp., *Babesia* spp. and *L. infantum* infections are widespread in wild carnivores from Spanish Mediterranean ecosystems, and provided strong evidence that there is an increased risk of *L. infantum* infection in wildlife infected with *Babesia* spp. and *Hepatozoon* spp. *H. canis* is highly prevalent in foxes and so is *H. martis* in beech martens. Furthermore, it was shown for the first time that *H. canis* infects beech martens, *H. martis* infects wild cats, and genets are a target species for *Sarcocystis* spp. and may act as a definite host of *S. cymruensis*. *B. vulpes* is highly prevalent in foxes and so is *Babesia* sp. badger type A in badgers. Moreover, *Babesia* sp. also infects wild cats in Spain. Therefore, wildlife mammals may be an important source of *Babesia* spp. and *Hepatozoon* spp. infections for domestic animals.

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Supporting information

Table S1. Estimates of evolutionary divergence (%) between *Babesia* spp. sequences from this (►) and other studies, using the Kimura 2-parameter model.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	►***Babesia* sp. isolate BSP1 (badger, Spain)**																		
2	►***Babesia* sp. isolate BSP3 (badger, Spain)**	0.0																	
3	KT223484 *Babesia* sp. badger type A (badger, Spain)	0.0	0.0																
4	MT077002 *Babesia* sp. (badger, United Kingdom)	0.0	0.0	0.0															
5	KT223485 *Babesia* sp. badger type B (badger, Spain)	5.9	5.8	2.9	3.3														
6	MG799846 *Babesia* sp. badger type B (badger, China)	6.3	6.2	2.9	3.6	0.2													
7	MT509981 *Babesia vulpes* (dog, Russia)	2.7	2.2	0.9	1.5	2.1	2.5												
8	MK585200 *Babesia vulpes* (dog, Spain)	2.0	1.7	2.2	1.4	3.4	3.6	0.2											
9	MK957183 *Babesia vulpes* (fox, Iraq)	2.0	1.7	1.3	1.5	3.9	4.2	0.2	0.0										
10	►***Babesia vulpes* isolate BV1 (fox, Spain)**	2.0	2.3	2.0	2.0	4.8	5.1	0.3	0.0	0.0									
11	►***Babesia vulpes* isolate BV2 (fox, Spain)**	3.1	2.2	2.2	2.7	5.2	5.5	0.0	0.3	0.3	0.4								
12	►***Babesia vulpes* isolate BV3 (fox, Spain)**	3.1	2.2	2.2	2.7	5.2	5.5	0.0	0.3	0.3	0.4	0.0							
13	KJ956782 *Babesia* sp. MML-2014 (fox, Israel)	21.5	21.1	13.6	12.2	15.2	16.2	12.8	15.4	16.4	22.3	21.4	21.0						
14	MK957184 *Babesia* sp. (golden jackal, Iraq)	21.5	21.1	16.9	17.0	21.8	22.2	17.1	17.4	17.7	22.3	21.4	21.0	0.0					
15	►***Babesia* sp. isolate BSP2 (wild cat, Spain)**	20.9	25.3	20.9	20.9	26.2	26.8	21.3	21.8	21.8	21.8	25.7	25.1	1.3	1.3				
16	KF270672 *Babesia* sp. (hyena, Zambia)	23.5	22.8	9.7	12.8	11.4	11.6	9.9	15.8	17.3	24.4	23.2	22.7	3.3	4.4	4.1			
17	KX218431 *Babesia* sp. (African lion, Botswana)	22.7	21.3	14.2	13.0	15.5	15.2	13.1	15.8	17.6	24.2	22.1	21.7	2.4	4.9	5.3	2.8		
18	GQ411405 *Babesia lengau* (cheetah, South Africa)	22.7	21.3	14.0	12.4	15.3	16.0	12.8	15.4	17.2	24.2	22.1	21.7	3.0	4.9	5.3	2.5	0.1	

Table S2. Estimates of evolutionary divergence (%) between *Hepatozoon canis* sequences from this (►) and other studies, using the Kimura 2-parameter model.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	►***Hepatozoon canis* isolate HC1 (fox. Spain)**																		
2	►***Hepatozoon canis* isolate HC2 (fox. Spain)**	0.9																	
3	►***Hepatozoon canis* isolate HC3 (beech marten. Spain)**	0.9	0.0																
4	►***Hepatozoon canis* isolate HC4 (fox. Spain)**	0.9	0.6	0.6															
5	►***Hepatozoon canis* isolate HC5 (fox. Spain)**	1.2	0.9	0.9	0.3														
6	►***Hepatozoon canis* isolate HC6 (fox. Spain)**	0.0	0.0	0.0	0.3	0.6													
7	►***Hepatozoon canis* isolate HC7 (fox. Spain)**	0.3	0.6	0.6	0.0	0.3	0.3												
8	►***Hepatozoon canis* isolate HC8 (fox. Spain)**	0.0	0.6	0.6	0.3	0.6	0.0	0.0											
9	►***Hepatozoon canis* isolate HC9 (fox. Spain)**	0.0	0.9	0.9	0.6	0.9	0.0	0.0	0.0										
10	►***Hepatozoon canis* isolate HC10 (fox. Spain)**	0.6	0.9	0.9	0.3	0.3	0.3	0.0	0.0	0.3									
11	►***Hepatozoon canis* isolate HC11 (fox. Spain)**	0.3	0.6	0.6	0.3	0.6	0.0	0.0	0.0	0.3	0.0								
12	►***Hepatozoon canis* isolate HC12 (fox. Spain)**	0.0	0.3	0.3	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0							
13	►***Hepatozoon canis* isolate HC13 (fox. Spain)**	0.9	0.9	0.9	0.3	0.0	0.6	0.3	0.3	0.6	0.0	0.3	0.3						
14	►***Hepatozoon canis* isolate HC14 (beech marten. Spain)**	0.8	0.0	0.0	0.8	1.7	0.0	0.8	0.0	0.8	0.8	0.0	0.0	1.7					
15	►***Hepatozoon canis* isolate HC15 (fox. Spain)**	2.6	1.7	1.7	0.8	0.0	1.7	0.8	0.9	1.7	0.0	0.8	0.9	0.0	0.4				
16	KP216425 *Hepatozoon canis* (fox. Bosnia and Herzegovina)	0.9	0.9	0.9	0.3	0.0	0.6	0.3	0.3	0.6	0.0	0.3	0.3	0.0	1.0	0.0			
17	MN791089 *Hepatozoon canis* (wolf. Germany)	1.2	0.9	0.9	0.3	0.0	0.6	0.3	0.6	0.9	0.3	0.6	0.3	0.0	0.7	0.0	0.0		
18	MN628329 *Hepatozoon canis* (dog. India)	0.9	1.2	1.2	0.6	0.3	0.6	0.3	0.3	0.6	0.0	0.3	0.3	0.0	0.9	0.0	0.0	0.2	

Table S3. Estimates of evolutionary divergence (%) between *Hepatozoon felis* and *H. martis* sequences from this (►) and other studies, using the Kimura 2-parameter model.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	►***Hepatozoon felis*** **isolate HF1 (wild cat, Spain)**																					
2	►***Hepatozoon felis*** **isolate HF2 (wild cat, Spain)**	0.9																				
3	►***Hepatozoon felis*** **isolate HF3 (wild cat, Spain)**	0.0	0.8																			
4	AY628681 *Hepatozoon felis* (cat, Spain)	0.0	0.9	0.0																		
5	MG386483 *Hepatozoon felis* (cat, Angola)	0.0	0.9	0.0	0.0																	
6	MN793001 *Hepatozoon ingwe* (African leopard, South Africa)	0.6	0.3	0.7	0.7	0.6																
7	MF614156 *Hepatozoon felis* (wild cat, Bosnia and Herzegovina)	0.9	0.6	0.5	0.9	0.9	0.4															
8	MN793002 *Hepatozoon luiperdjie* (African leopard, South Africa)	2.8	2.2	1.1	1.3	2.8	0.8	1.5														
9	KX757032 *Hepatozoon silvestris* (wild cat, Bosnia and Herzegovina)	3.8	3.4	2.6	2.1	3.8	1.4	2.6	1.3													
10	►***Hepatozoon martis*** **isolate HM1 (wild cat, Spain)**	2.5	0.9	4.4	2.5	2.5	1.8	2.2	2.8	3.8												
11	►***Hepatozoon martis*** **isolate HM2 (beech marten, Spain)**	2.5	0.9	4.4	2.5	2.5	1.8	2.2	2.8	4.1	0.3											
12	►***Hepatozoon martis*** **isolate HM3 (beech marten, Spain)**	3.1	1.5	6.3	3.1	3.1	2.5	2.8	3.4	4.4	0.3	0.6										
13	►***Hepatozoon martis*** **isolate HM4 (badger, Spain)**	3.1	1.5	6.3	3.1	3.1	2.5	2.8	3.4	4.4	0.3	0.6	0.0									
14	►***Hepatozoon martis*** **isolate HM5 (badger, Spain)**	6.3	4.4	3.7	3.7	6.3	2.6	3.6	2.6	3.5	0.8	1.7	0.0	0.0								
15	►***Hepatozoon martis*** **isolate HM6 (beech marten, Spain)**	6.3	4.4	3.7	3.7	6.3	2.6	3.6	2.6	3.5	0.8	1.7	0.0	0.0	0.0							
16	KU198330 *Hepatozoon* sp. (badger, Spain)	3.1	1.5	3.7	2.8	3.1	1.5	1.8	1.8	3.0	0.3	0.6	0.0	0.0	0.0	0.0						
17	EF222257 *Hepatozoon* sp. (pine marten, Spain)	3.1	1.5	3.5	4.0	3.1	1.3	1.8	1.5	3.0	0.3	0.6	0.0	0.0	0.8	0.8	0.2					
18	MG136687 *Hepatozoon martis* (pine marten, Bosnia and Herzegovina)	3.1	1.5	3.7	2.8	3.1	1.5	1.8	1.8	2.9	0.3	0.6	0.0	0.0	0.0	0.0	0.0	0.2				
19	MG136688 *Hepatozoon martis* (beech marten, Croatia)	3.1	1.5	3.7	2.8	3.1	1.5	1.8	1.8	2.9	0.3	0.6	0.0	0.0	0.0	0.0	0.0	0.2	0.0			
20	FJ595128 *Hepatozoon* sp. (Japanese marten, Japan)	4.5	1.9	3.5	3.9	4.5	2.1	2.8	2.1	3.9	0.9	0.5	0.5	0.5	0.2	0.2	0.3	1.0	0.3	0.3		
21	MH656728 *Hepatozoon martis* (European polecat, Netherlands)	4.8	2.3	3.7	4.0	4.8	2.3	3.1	2.3	3.6	0.5	0.9	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.3	

Table S4. Estimates of evolutionary divergence (%) between *Cytauxzoon* spp. sequences from this (►) and other studies, using the Kimura 2-parameter model.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	► ***Cytauxzoon* sp. isolate CYT1 (wild cat, Spain)**															
2	AY485690 *Cytauxzoon manul* (Pallas's cat, Mongolia)	0.0														
3	KT361070 *Cytauxzoon* sp. (Eurasian lynx, Romania)	0.0	2.5													
4	AY309956 *Cytauxzoon* sp. (cat, Spain)	0.3	0.7	2.7												
5	KU710344 *Cytauxzoon manul* (cat, Portugal)	0.0	0.1	0.4	0.1											
6	MN629916 *Cytauxzoon* sp. (cat, Germany)	0.0	0.2	2.7	0.2	0.0										
7	EU622908 *Cytauxzoon* sp. (cat, France)	0.0	0.3	2.3	0.4	0.1	0.1									
8	AY496273 *Cytauxzoon* sp. (Iberian lynx, Spain)	0.0	0.4	2.5	1.0	0.0	0.1	0.8								
9	KT223486 *Cytauxzoon* sp. (wild cat, Spain)	0.0	0.3	0.3	0.3	0.0	0.0	0.3	0.0							
10	HM146424 *Cytauxzoon* sp. (cat, Italy)	0.0	0.3	0.3	0.3	0.0	0.0	0.3	0.0	0.0						
11	MT904044 *Cytauxzoon europaeus* (wild cat, Luxembourg)	0.0	0.2	2.3	0.5	0.0	0.0	0.0	0.1	0.0	0.0					
12	MT904033 *Cytauxzoon otrantorum* (wild cat, Romania)	0.0	0.2	2.3	0.5	0.0	0.0	0.0	0.1	0.0	0.0	0.0				
13	MT904029 *Cytauxzoon banethi* (wild cat, Romania)	1.0	0.4	2.6	0.8	0.5	0.5	0.4	0.5	1.0	1.1	0.4	0.4			
14	AY679105 *Cytauxzoon felis* (cat, USA)	7.6	3.7	6.7	4.2	4.3	4.7	3.9	4.7	7.8	7.9	3.8	3.5	3.2		
15	GU903911 *Cytauxzoon felis* (ocelot, Brazil)	7.6	3.2	6.6	3.8	4.4	4.7	3.4	3.4	8.1	8.2	3.6	3.4	3.1	0.4	

Table S5. Estimates of evolutionary divergence (%) between *Sarcocystis* spp. sequences from this (►) and other studies, using the Kimura 2-parameter model.

		1	2	3	4	5	6	7	8	9	10	11
1	**►*Sarcocystis* sp. isolate SC1 (genet, Spain)**											
2	MT372786 *Sarcocystis cymruensis* (brown rat, China)	0.0										
3	MG564723 *Sarcocystis cymruensis* (brown rat, West Indies)	0.0	0.0									
4	KC877996 *Sarcocystis muris* (cat, Egypt)	0.0	0.0	0.0								
5	AY015111 *Sarcocystis rodentifelis* (vole, Lithuania)	0.0	0.1	0.2	0.2							
6	M64244 *Sarcocystis muris* (mouse, USA)	0.6	1.9	0.7	0.6	0.6						
7	KT184371 *Sarcocystis neurona* (raccoon, Canada)	3.2	1.7	1.9	1.6	1.5	1.9					
8	KT207459 *Sarcocystis speeri* (opossum, Argentina)	3.2	2.9	2.3	2.0	1.9	1.9	0.0				
9	MF596237 *Sarcocystis arctica* (fox, Spain)	3.9	3.4	2.7	2.4	2.1	1.9	1.0	0.9			
10	HQ709143 *Sarcocystis* sp. (fisher, Pennsylvania)	3.9	2.9	2.9	2.9	3.0	2.9	1.2	1.1	0.1		
11	KJ851560 *Sarcocystis* sp. (rodent, Malaysia)	0.3	0.3	0.3	0.3	0.3	0.8	3.2	3.2	3.5	3.8	

Table S6. Relationship between infections with different *Babesia* spp., *Hepatozoon* spp. and *L. infantum*.

			Babesia vulpes		***Babesia* sp. type A**		**Other *Babesia* sp.**		***Hepatozoon canis***		***Hepatozoon martis***		***Hepatozoon felis***		***Leishmania infantum***	
	Infected	**No.**	**% +ve.[1]**	**p-value**	**% +ve.**	**p-value**	**% +ve.**	**p-value**	**% +ve.**	**p-value**	**% +ve.**	**p-value**	**% +ve.**	**p-value**	**% +ve.**	**p-value**
Foxes																
Babesia	Yes	57	-	-	-	-	-	-	89	0.7059	-	-	-	-	37	0.0511
vulpes	No	32	-		-		-		94		-		-		16	
Hepatozoon	Yes	81	63	0.7059	-	-	-	-	-	-	-	-	-	-	32	0.0988
canis	No	8	75		-		-		-		-		-		0	
Leishmania	Yes	26	81	0.0511	-	-	-	-	100	0.0988	-	-	-	-	-	-
infantum	No	63	57		-		-		87		-		-		-	
Badgers																
Babesia sp.	Yes	14	-	-	-	-	-	-	-	-	14	1.0000	-	-	14	0.4928
type A	No	10	-		-		-		-		10		-		0	
Hepatozoon	Yes	3	-	-	67	1.0000	-	-	-	-	-	-	-	-	0	1.0000
martis	No	21	-		57		-		-		-		-		10	
Leishmania	Yes	2	-	-	100	0.4928	-	-	-	-	0	1.0000	-	-	-	-
infantum	No	22	-		55		-		-		14		-		-	
Beech martens																
Hepatozoon	Yes	2	-	-	-	-	-	-	-	-	0	0.0250	-	-	50	0.2417
canis	No	14	-		-		-		-		93		-		7	
Hepatozoon	Yes	13	-	-	-	-	-	-	0	0.0250	-	-	-	-	8	0.3500
martis	No	3	-		-		-		67		-		-		33	
Leishmania	Yes	2	-	-	-	-	-	-	50	0.2417	50	0.3500	-	-	-	-
infantum	No	14	-		-		-		7		86		-		-	
Wild cats																
Babesia sp.	Yes	1	-	-	-	-	-	-	-	-	0	1.0000	0	0.4000	-	-
	No	4	-		-		-		-		25		75		-	
Hepatozoon	Yes	1	-	-	-	-	0	1.0000	-	-	-	-	0	0.4000	-	-
martis	No	4	-		-		25		-		-		75		-	
Hepatozoon	Yes	3	-	-	-	-	0	0.4000	-	-	0	0.4000	-	-	-	-
felis	No	2	-		-		50		-		50		-		-	

[1] PCR-positive

CHAPTER 3

Exposure to *Phlebotomus perniciosus* sandflies and risk of Toscana and Sicilian phleboviruses and *Leishmania infantum* infection in blood donors from southeast Spain

Abstract

The serological response to *Phlebotomus perniciosus* sand fly salivary gland homogenate and recombinant protein rSP03B and the prevalence of sand fly transmitted Toscana and Sicilian viruses and *Leishmania infantum* was investigated in 670 blood samples from 575 donors in southeast Spain, sampled in 2017 and 2018. Antibodies against *P. perniciosus* salivary proteins in humans have not been analyzed before, and 98% of donors were seropositive to salivary gland homogenate and/or rSP03B assays but correlation in the (logarithmic) antibody optical density (LOD) between tests was relatively low (ρ = 0.39). Toscana virus, Sicilian virus and *L. infantum* seroprevalences were 26%, 0% and 1%, respectively, and *L. infantum* PCR prevalence was 2%. Coinfection between Toscana virus and *L. infantum* was detected in 1% of donors. Salivary gland homogenate LOD was positively associated to Toscana virus seroprevalence and *L. infantum* PCR-status. Toscana virus seroprevalence was highest in older people from rural areas sampled in 2017 ($p<0.05$) instead, *L. infantum* was more prevalent in middle aged donors from periurban settings but differences were not statistically significant. The fact that areas with highest Toscana virus and *L. infantum* prevalence did not coincide in most instances suggests that transmission cycles for these pathogens in the study area are not dependant on each other. The absence of a relationship between rSP03B LODs and Toscana virus and *L. infantum* infections questions its value as a diagnostic marker for these infections in humans. The clinical and epidemiological implications of Toscana virus and *L. infantum* prevalence are discussed.

Introduction

Human leishmaniosis (HumL) and phleboviroses are vector-borne diseases transmitted by phlebotomine sandflies that are endemic in Mediterranean countries. In Spain, HumL is caused by the protozoan *Leishmania infantum* (Trypanosomatidae), and typical clinical presentations include visceral leishmaniosis (VL) and cutaneous leishmaniosis (CL) (WHO, 2021). *Phlebovirus* (*Bunyaviridae*) infections and/or antibodies reported in Spain include Toscana virus (TOSV), Sandfly Fever Sicilian virus (SFSV), Sandfly Fever Naples virus (SFNV) and Granada virus (GRV) (Mendoza-Montero et al., 1998; Navarro-Marí et al., 2013), although clinical cases have only been described for TOSV, namely meningitis

and meningoencephalitis (Echevarría et al., 2003; Martínez-García et al., 2007; De Ory et al., 2009; Cardeñosa et al., 2013). However, subclinical infections by *L. infantum* and phleboviruses in people are common. The estimated *L. infantum* antibody and PCR prevalences in blood samples from healthy humans in Spain range from 0-56% and 0-22%, respectively (Aliaga et al., 2019; Pérez-Cutillas et al., 2015; Chitimia et al., 2011; Riera et al., 2004). Similarly, TOSV and SFSV seroprevalences range from 5-26% and 0-2%, respectively (Cardeñosa et al., 2013; de Ory et al., 2009; Echevarría et al., 2003; Mendoza-Montero et al., 1998; Sanbonmatsu-Gámez et al., 2005).

However, diagnosing subclinical sand fly-borne infections is challenging. Phleboviral infections have a short viremic period (Charrel & de Lamballerie, 2013) and prevalence estimations rely on serological tests, the most specific being the virus seroneutralization test (VNT) (Ayhan & Charrel, 2017). Most *L. infantum* infections do not elicit a strong humoral response and the real-time polymerase chain reaction (PCR) test in lymphoid tissue samples is the most sensitive technique (Caldas et al., 2020; Reale et al., 1999). Moreover, the risk of infection with these pathogens is likely related to sandfly biting rates which may be assessed by investigating circulating antibodies against sandfly salivary antigens (Kostalova et al., 2015; Martín-Martín, Molina & Jiménez, 2015). Among the twelve *Phlebotomus* species present in Spain, *Phlebotomus perniciosus*, is the most abundant and widely distributed *L. infantum* vector (Lucientes et al., 2005). It is also involved in *Phlebovirus* transmission amid a wider range of other sandfly species (Dehghami et al., 2021). Sandfly salivary gland homogenates (SGH) from this species have been successfully used as the antigen source in antibody enzyme-linked immunosorbent assay (ELISA) tests in dogs, cats, mice and leporids (Drahota et al., 2014; Martín-Martín et al., 2014; Kostalova et al., 2016; Pereira et al., 2019) but not in humans. However, SGHs are cumbersome to obtain as they require dissection of individual sandflies from laboratory colonies and may lack species-specificity (Martín-Martín, Molina & Jiménez, 2015; Rohousova et al., 2006). To overcome this, recombinant salivary gland proteins provided a good alternative to SGH in studies in dogs, mice and leporids, particularly the 43-kDa yellow-related protein rSP03B (Drahota et al., 2014; Kostalova et al., 2015; Martín-Martín et al., 2014). No studies have investigated antibodies in humans against *P. perniciosus* salivary proteins. Marzouki et al. (2015) analyzed antibodies against SGH and the recombinant protein PpSP32 from *P. papatasi* in Tunisian people observing moderate correlation between tests.

Understanding the prevalence, distribution and risk factors of sandfly biting activity and its relationship with *L. infantum* and *Phlebovirus* infection is important from a prevention and control perspective. Here we investigated human exposure to *P. perniciosus* for the first time, by analyzing the antibody response to SGH and rSP03B proteins, and the prevalence of *L. infantum*, TOSV and SFSV. The target population were blood donors from Murcia Region in southeast Spain, where previous studies revealed subclinical *L. infantum* infection in this cohort (Chitimia et al., 2011; Pérez-Cutillas et al., 2015).

Materials and methods

Study population

The study included 670 human blood samples taken in January-March 2017 and September-November 2018 from 575 voluntary donors (95 donors provided samples both years), from 13 rural villages and periurban towns of Murcia Region (Figs. 1 and 2). A questionnaire survey was conducted to gather information on donor`s demographical data, residence, work, dog ownership, use of insecticides and knowledge about leishmaniosis.

Salivary gland homogenate (SGH) and recombinant protein rSP03B

Four to six days-old female *P. perniciosus* sandflies kept in laboratory colonies at Charles University, Prague, were dissected for salivary gland extraction (Volf & Volfova, 2011). Salivary glands were pooled in groups of 20 together with 20µL of Tris-NaCl buffer (20 mm Tris, 150 mm NaCl; pH 7.4) and submitted to three consecutive freeze-thaw cycles to obtain SGH, that were then stored at −20ºC until used (Kostalova et al., 2015).

The recombinant *P. perniciosus* 43-kDa yellow protein (rSP03B, GenBank accession no. DQ150622) was manufactured by Apronex s.r.o. (Prague, Czech Republic) following the protocol described by Drahota et al. (2014). The concentrations of these proteins were quantified by the Lowry method (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) following the manufacturer’s protocol.

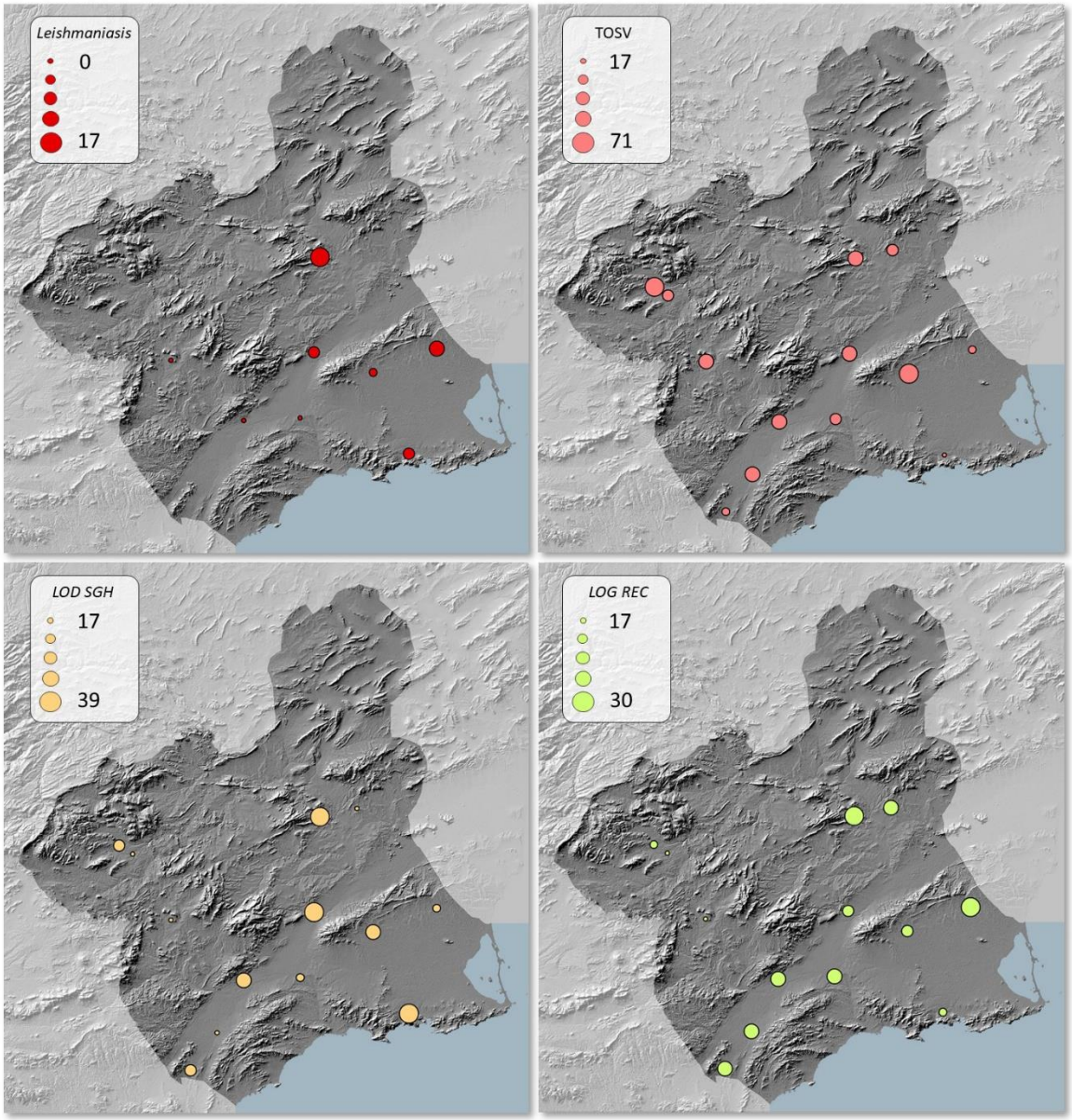

Figure 1. Distribution of *Leishmania infantum* and Toscana *Phlebovirus* prevalences and median LODs against salivary gland homogenate and rSP03B, according to blood donation centre in the year 2017.

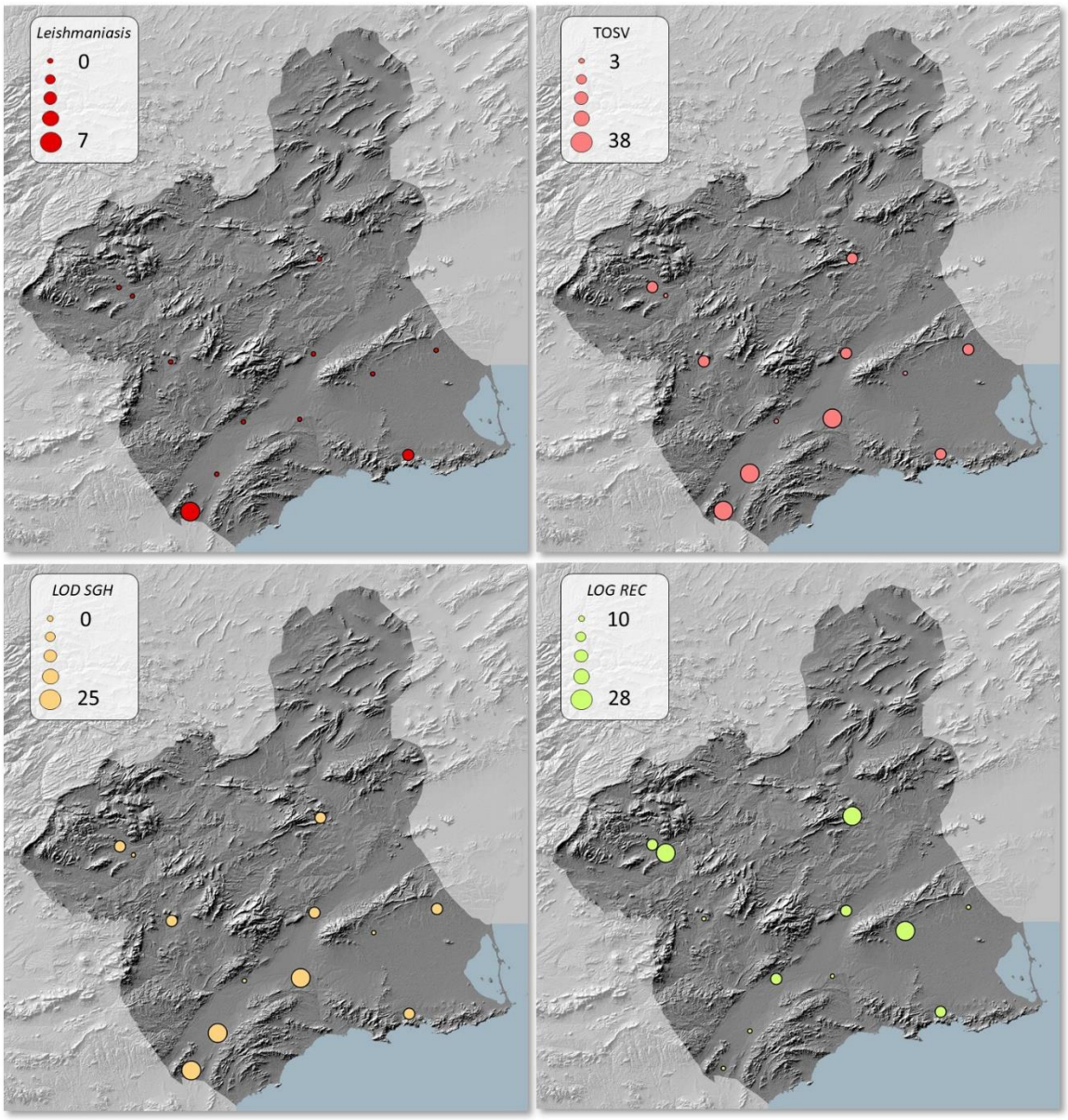

Figure 2. Distribution of *Leishmania infantum* and Toscana *Phlebovirus* prevalences and median LODs against salivary gland homogenate and rSP03B, according to blood donation centre in the year 2018.

Detection of IgG anti-SGH and anti-rSP03B antibodies

The ELISA protocol described by Kostalova et al. (2015) was used for analyzing specific IgG anti-saliva antibodies, with slight modifications. Sand fly salivary gland pools were mixed, and a single homogenate was used for antigen coating all microtiter ELISA plates, to limit between-plate differences. Microtiter plate antigen coating was performed with either SGH (40 ng per well, equivalent to 0.2 gland) or rSP03B (0.2 µg per well). The ELISA/ELISPOT Diluent 1X (eBioscience™) was employed as the blocking solution. Human sera were diluted to 1:100 in 1X ELISA diluent. Secondary antibodies (Goat Anti-Human IgG Antibody, HRP conjugate, Sigma-Aldrich) were diluted to 1:5000 (for SGH) and 1:2500 (for rSP03B) in PBS-Tw. An ELx800 Absorbance Microplate Reader (BioTek, USA) was used to measure optical densities (ODs) at 490 nm. Each serum sample was tested in duplicate, and each plate contained four methodical controls (MC) and a positive control from rabbit sera. For the latter, goat anti-Rabbit IgG antibody, peroxidase conjugate (Sigma Aldrich), was used at 1:2500 for rabbit sera. Due to the unavailability of human positive and negative controls, a preliminary 96 well plate with a random set of samples was analyzed and those with the highest and lowest ODs were selected as human positive and negative controls in subsequent plates. All serum samples were processed by the same analyst in the same laboratory in two days in 2019 for SGH and 2020 for rSP03B.

Standardized optical densities (SOD) of ELISA antibodies against salivary gland proteins across plates were calculated using the following formula (Sanchez et al., 2002): $SOD = (OD_S - OD_{NC}) / (OD_{PC} - OD_{NC})$ (S: sample; NC: negative control; PC: positive control). After approximating negative values to zero, SODs+1 were decimal log-transformed, multiplied by 100 and named LODs. A sample LOD was obtained by calculating the mean LOD of both readings. Human's serological status (seropositive or seronegative) could not be estimated since no cut-off has been established for these antigens in humans.

Detection of anti-TOSV and anti-SFSV neutralizing antibodies

The virus microneutralization assay (VNT) was performed to assess TOSV and SFSV neutralizing antibody titers (Sakhria et al., 2013) in plasma samples collected in 2017 (n=350) and only for TOSV in those from 2018 (n=320), since no SFSV-positives were detected in 2017. Briefly, assays were performed in 96-well plates, 50 µL of serial plasma dilutions (preheated at 56ºC for 30 minutes) were added to the wells to obtain final plasma

dilutions ranging from 1:10 to 1:160, followed by 50 µL of infectious virus at 100 TCID50 and plates were incubated at 37°C with 5% CO_2 for one hour. Subsequently, a 100 µL suspension of Vero cells (ATCC CCL81) incorporating approximately $2x10^5$ cells/mL, 5% fetal bovine serum, 1% Penicillin-Streptomycin, 1% L-Glutamine and 1% Kanamycin-enriched MEM medium (200 mM), were added to each well. The presence or absence of cytopathic effect was evaluated in an inverted microscope after incubating plates at 37ºC in a 5% CO_2 atmosphere for five days for TOSV and for six days for SFSV. Positive samples were those with no cytopathic effect at 1:40 or greater serum dilutions. Control wells, containing virus + Vero cells, Vero cells only, and plasma samples + Vero cells, were included in each plate.

Detection of IgG and IgM anti-*L. infantum*

The "LEISHMANIA ELISA IgG+IgM" kit (Vircell ®) was used to detect *L. infantum*-specific serum antibodies. According to the protocol, the antibody-index (*I*=sample OD/cut-off OD) was calculated for all samples and samples were considered positive when $I > 1.1$, doubtful if $I = 0.9\text{-}1.1$ and negative if $I < 0.9$.

DNA extraction and *L. infantum*-PCR amplification

Whole blood DNA was extracted using the Blood DNA Purification Kit in the Maxwell® 16 semi-automated nucleic acid purification robot (Promega). DNA was extracted individually for 550 donors and in pools of four samples for 120 individuals. The latter included samples from 5 blood donation centers taken in 2017. DNA concentration and purity were analyzed using NanoDrop 2000® spectrophotometer (Thermo Fischer Scientific). *Leishmania infantum* DNA was investigated by a quantitative PCR (qPCR) that amplified an approximately 120 base pair (bp) fragment of the kinetoplast DNA (kDNA) minicircle (Francino et al., 2006), following Dantas-Torres et al. (2017). The rtPCR threshold cycle (CT) was considered as a semi-quantitative measure of parasite DNA amplified. Samples with $CT \geq 40$ were considered negative.

Data mapping and statistical analysis

Blood donors' residence addresses were mapped and spatial autocorrelation among *L. infantum* and *Phlebovirus* positives and negatives was evaluated using Moran´s I Index. Spearman's rank test was used to assess correlations between anti-SGH and anti-rSP03B

antibody levels (Kirkwood & Sterne, 2003; Prion & Haerling, 2014). Associations between the donor's *Leishmania*-PCR and TOSV serological status (binary variables) and *Leishmania*, SGH and rSP03B ELISA LODs (continuous variables) with questionnaire independent variables were assessed using Fisher's exact and the Kruskal-Wallis test, respectively. Moreover, the independent contribution of independent variables significantly associated with the outcome variables in the binary analyses, was assessed using Logistic and Negative binomial regression models for categorical and continuous outcome variables, respectively. The R statistical software (http://cran.r-project.org/) was used for all except for the autocorrelation analysis, which was performed in ArcGISv.10 (ESRI) geographical information system. Differences were considered statistically significant for $p<0.05$ and marginally significant for $p<0.10$, for a two-tailed test. Donors that had not been individually tested for *Leishmania*-PCR were not included in these analyses, except for one donor who was positive to *L. infantum*-ELISA.

Results

ELISA antibody LODs against *P. perniciosus* SGH and rSP03B salivary proteins

Median LODs were 18 (range: 0-67) for SGH and 18 (range: 0-90) for rSP03B *P. perniciosus* salivary gland proteins (Table 1). It was higher in 2017 compared to 2018 and varied significantly between blood donation centers (Fig. 3). Correlation between SGH and rSP03B LODs was weak overall (ρ = 0.39, $p<0.01$) (Fig. 4), and moderate in 2018 (ρ = 0.50, $p<0.01$). However, it was relatively strong when comparing blood donation center medians in 2018 (ρ = 0.66, $p<0.05$). Among the 95 donors sampled twice, in 2017 and 2018, SGH LODs decreased in 93% and increased in 7% of donors. Similar percentages for rSP03B were 66% and 34%, respectively.

SGH and rSP03B LODs decreased with age (significantly for rSP03B only, $p<0.05$) and were higher in females, in donors working permanently indoors, and owning dogs sleeping only indoors and tested for *Leishmania* (significant for SGH only, $p<0.05$) (Table 1). Additionally, rSP03B LODs were higher among non-Spanish donors, those living in flats compared to country houses and those owning companion dogs. Likewise, SGH LODs increased with the municipal census of the blood donation center and were higher among owners of dogs being presently treated for leishmaniosis (Table 1). Antibody LODs were

not associated with insecticide use or with other donor's demographic and social variables (Table 1S).

In the negative binomial regression model, SGH LOD was independently and significantly associated with the blood donation center, year 2017 and owning a dog tested for *Leishmania* ($p<0.05$), and marginally with being female ($p<0.10$) (Table 2S). Similarly, rSP03B LOD was positively associated to the blood donation center and young donors, living in house distinct from country houses and owning a pet dog ($p<0.05$), and was marginally higher in 2017 ($p<0.10$) (Table 3S).

Table 1. Median (range) anti-SGH and anti-rSP03B LOD values and *L. infantum* and TOSV prevalences in blood donors from Murcia Region according to sampling period, blood donation centre, and donor's gender, age, nationality, residential, work and dog ownership features, leishmaniosis awareness, and TOSV serological status.

Variable	N	Median (range) SGH	*P* value	Median (range) rSP03B	*P* value	N	% *L. infantum* positive (qPCR + ELISA)	*P* value	N	% TOSV seropositive	P value
Total	**670**	**18 (0-67)**		**18 (0-90)**		**550**	**3.6**		**659**	**26**	
Sampling period											
Feb-Mar 2017	350	27 (0-67)*	<0.0001	20 (0-58)*	0.0003	230	7.4*	<0.0001	339	38*	<0.0001
Sep-Nov 2018	320	5 (0-53)		17 (0-90)		320	0.9		320	14	
Gender											
Male	349	16 (0-67)	0.0030	18 (0-73)	0.0030	294	4.8	0.1711	340	29	0.0630
Female	321	20 (0-64)*		20 (0-90)*		256	2.3		319	23	
Age (years)											
18-34	175	20 (0-59)	0.1304	22 (0-88)*	0.0002	142	2.8	0.7945	174	21	<0.0001
35-50	340	18 (0-67)		18 (0-90)		282	4.3		331	22	
50-65	155	16 (0-63)		17 (0-82)		126	3.2		154	40*	
Nationality											
Spanish	653	18 (0-67)	0.2255	18 (0-90)	0.0139	538	3.5	0.3618	642	26	0.5795
Other	17	25 (0-51)		28 (6-82)*		12	8.3		17	18	
Residential municipality census											
430-1500	290	14 (0-58)	<0.0001	18 (0-90)	0.3581	220	2.3	0.2824	289	28	0.0410
1720-2762	200	17 (0-59)		19 (0-81)		150	5.3		198	30*	
3967-10285	180	30 (0-67)*		18 (0-65)		180	3.9		172	19	
Residential setting											
Urban	276	18 (0-67)	0.5987	18 (0-88)	0.4810	344	4.4	0.3474	383	22	0.0068
Rural	388	19 (0-64)		18 (0-90)		200	2.5		270	32*	
Home type											
Flat	121	20 (0-64)	0.0757	20 (0-88)*	0.0397	104	4.8	0.7033	119	23	0.1248
Semi-detached	479	18 (0-67)		18 (0-82)		390	3.8		472	26	

Table 1 (continued). Median (range) anti-SGH and anti-rSP03B LOD values and *L. infantum* and TOSV prevalences in blood donors from Murcia Region according to sampling period, blood donation centre, and donor's gender, age, nationality, residential, work and dog ownership features, leishmaniosis awareness, and TOSV serological status.

Variable	N	Median (range) SGH	*P* value	Median (range) rSP03B	*P* value	N	% *L. infantum* positive (qPCR[1] + ELISA)	*P* value	N	% TOSV seropositive	P value
Detached	24	8 (0-51)		19 (0-90)		19	0.0		24	21	
Country house	43	16 (0-63)		15 (4-55)		35	0.0		41	41	
Working environment											
Outdoors	219	17 (0-67)	0.0023	17 (0-60)	0.0190	173	4.0	1.0000	215	35*	0.0067
Permanently indoors	339	21 (0-64)*		18 (0-90)*		286	4.2		332	24	
Working hours											
Exclude 20-24 h	568	18 (0-67)	0.5246	19 (0-90)	0.4178	469	3.4	0.5029	561	25	0.0553
Include 20-24 h	96	20 (0-53)		17 (0-82)		75	5.3		92	35	
Dog ownership											
Never	274	18 (0-64)	0.4504	18 (0-88)	0.0198	223	4.0	0.8173	271	24	0.2085
Now or in the past	393	18 (0-67)		20 (0-90)*		325	3.4		385	28	
Dog sleeping environment											
Indoors only	115	25 (0-58)*	<0.0001	21 (0-90)	0.0711	108	3.7	1.0000	111	23	0.2106
Outdoors or both	268	16 (0-67)		18 (0-76)		211	3.8		264	30	
Dog occupation											
Companion	280	19 (0-63)	0.1786	21 (0-90)*	0.0014	241	4.6	0.3060	272	25	0.0272
Work	100	16 (0-67)		16 (0-53)		76	1.3		100	37*	
Dog tested for *Leishmania*											
Yes	105	24 (0-67)*	0.0016	21 (0-79)	0.0647	92	3.3	1.0000	104	30	0.6032
No	256	17 (0-63)		19 (0-90)		212	3.8		249	27	
Dog treated for leishmaniosis											
Yes	15	30 (6-48)*	0.0050	24 (8-79)	0.4665	12	8.3	0.4758	31	39	0.0964
No	101	19 (0-67)		21 (0-81)		70	4.3		86	22	

*p<0.05, asterisk placed in the highest value.

[1] Only individual PCR results were used for statistical analysis

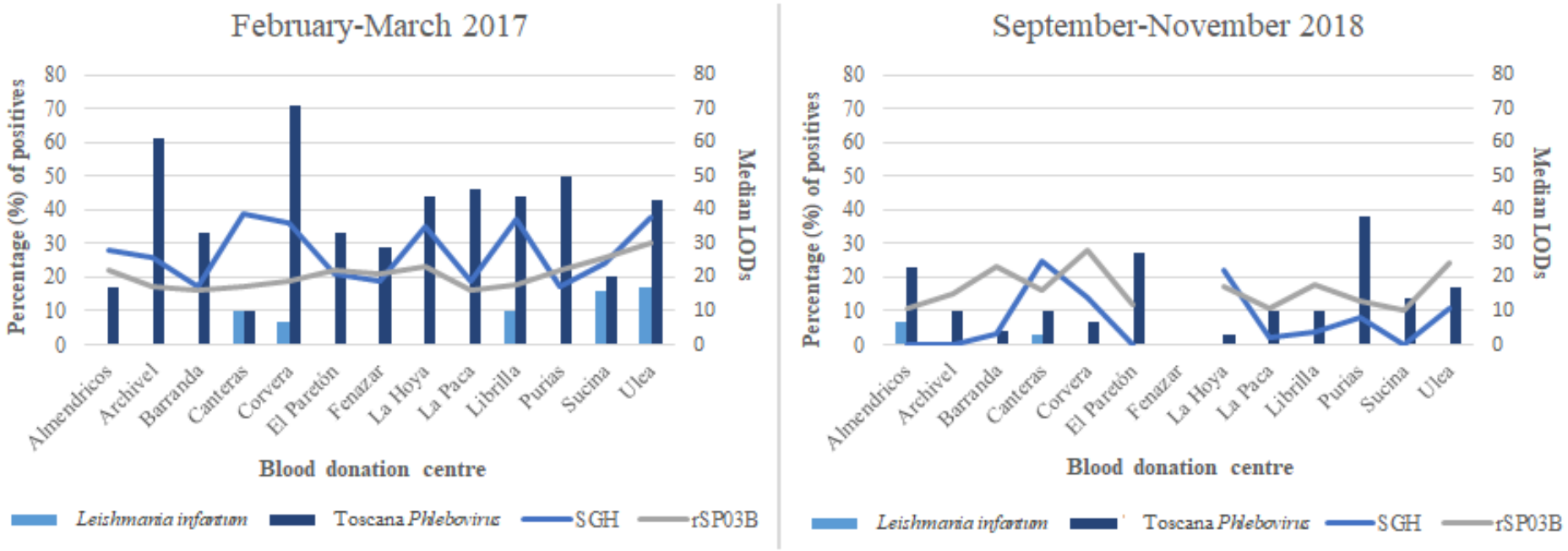

Figure 3. *Leishmania infantum* and Toscana *Phlebovirus* prevalences and median logarithmic optical dentsities (LODs) against salivary gland homogenate (SGH) and recombinant protein rSP03B according to year and blood donation centre.

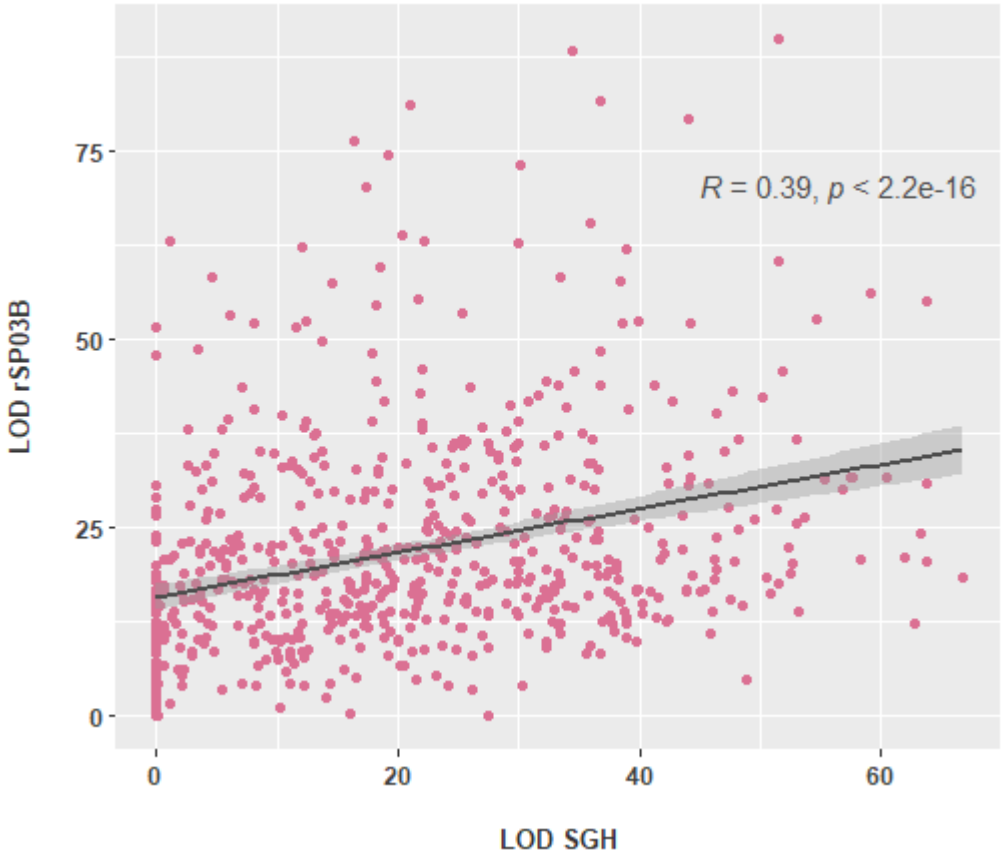

Figure 4. Spearman's correlation between individual human antibody log-transformed optical densities (LOD) against *Phlebotomus perniciosus* salivary gland homogenate (SGH) and rSP03B salivary protein.

Phlebovirus neutralizing antibody seroprevalence

Seroprevalence was 26% (173/659) for TOSV (Table 1) and 0% (0/350) for SFSV. Median (range) titre among TOSV seropositive individuals was 1/80 (1/40-1/160). Toscana seroprevalence was 38% in 2017 and 14% in 2018 ($p<0.05$) and virus positives came from every blood donation center, where seroprevalence ranged between 10% and 44% (Table 1). There was some evidence of spatial autocorrelation in donor' s TOSV status ($I=0.15$, $p<0.05$). Among the 95 donors sampled for TOSV in 2017 and in 2018, the percentage of donors seronegative both times was 56%, seropositive both times was 18%, seronegative in 2017 and seropositive in 2018 was 3% and seropositive in 2017 and seronegative in 2018 was 22%.

TOSV seroprevalence was highest in donors aged 50 years old or older, in rural residential settings, in blood donation centers from small municipalities (430-2762 inhabitants), in donors living in country houses, working outdoors, and owning working

dogs compared to pets ($p<0.05$), and it was marginally higher in males compared to females, in donors working during the time slot between 8 pm and midnight and owning dogs treated against leishmaniosis ($p<0.10$) (Table 1). The most parsimonious logistic regression model confirmed the association between the risk of being TOSV-positive and the blood donation center, year, age, working outdoors and having a working dog (Table 4S).

Median SGH LODs were significantly higher in TOSV-positive compared to negative donors (Table 2) and this relationship was observed across donation centers (Fig. 3). There was no association between rSP03B LODs and TOSV status.

Table 2. Median (range) anti-SGH and anti-rSP03B LOD values according to *L. infantum* and TOSV results.

	N	Median (range) anti-SGH LOD	*P* value	Median (range) anti-rSP03B LOD	*P* value
L. infantum PCR[1]					
Positive	13	23 (7-52)*	0.0316	19 (8-27)	0.7958
Negative	537	16 (0-67)		18 (0-90)	
L. infantum ELISA					
Positive	9	30 (4-58)	0.1867	18 (8-32)	0.9302
Negative	661	18 (0-67)		18 (0-90)	
L. infantum PCR+ELISA[1]					
Positive	20	23 (4-58)*	0.0087	19 (8-32)	0.7460
Negative	530	16 (0-67)		18 (0-90)	
TOSV					
Positive	173	24 (0-64)*	0.0002	19 (0-58)	0.7621
Negative	486	16 (0-67)		18 (0-90)	

[1] Only individual PCR results were used for statistical analysis

Leishmania infantum qPCR and ELISA prevalences

Fifteen DNA samples were positive to *Leishmania infantum*-qPCR, including 13/550 (2.4%) donors analyzed individually and 2/32 (6.3%) pools. Based on the latter, the estimated minimum *Leishmania*-PCR prevalence was 1.7% (2/120). The median (range) Ct in individually tested samples was 38.9 (range 36.9-39.5) and were 38.7-39.5 in 11 samples. Positives came from seven (54%) blood centres (Figs. 1-3), with prevalence ranging between 1.7% and 10.3%, and all except one positive sample were collected in 2017 ($p<0.05$). There were no significant differences in the qPCR prevalence according to

other variables although prevalence was marginally greatest (5.3%) among donors working during the 20:00-24:00 hours slot ($p<0.10$).

Overall *L. infantum* seroprevalence was 1.3% (9/670) and was not associated to the blood donation center or to any of the other explanatory variables. Only one individual was positive to both ELISA and qPCR. Six donors were coinfected with *L. infantum* and TOSV, representing 5% and 32% of TOSV and *L. infantum*-positive individuals, respectively.

Twenty out of the 550 (3.6%) individuals analyzed by ELISA and PCR were positive to either of those techniques. No explanatory variable was associated to *L. infantum* ELISA and/or qPCR positivity (Tables 1 and 1S). Median SGH were significantly higher in *L. infantum* qPCR and qPCR+ELISA positive donors compared to the negatives, whereas rSP03B LODs were not associated to donor´s *L. infantum* ELISA or PCR status (Table 2).

Discussion

This study provides first time population-based evidence that people living in rural and periurban areas in southeast Spain are exposed and develop antibodies to sandfly salivary proteins. Also, that TOSV but not SFSV infections, are widespread, with significant differences between years and areas, and greatest prevalence among older donors from rural areas. Higher median SGH LODs among TOSV seropositives compared to seronegatives suggests that the risk of infection is positively associated to sand fly biting rates, and that SGH is a suitable diagnostic marker for TOSV infection. Few donors were *L. infantum* positive and PCR prevalence was lower and CTs higher, indicating lower parasite loads, than in a similar study performed in the same areas in 2008 and 2010 (Pérez-Cutillas et al., 2015). Differences between studies could be related to variation in the annual and monthly incidence of infection. This would be supported by the fact that similarly marked differences in *L. infantum* and TOSV prevalence and in salivary gland LODs were observed between 2017 and 2018 in the present study.

This study investigated the diagnostic performance of rSP03B in humans for the first time. Correlation of the overall antibody response of *P. perniciosus* rSP03B with total SGH was only moderate. This is in contrast with the strong correlation found between these protein preparations when tested in dogs, mice and leporids (Drahota et al., 2014; Kostalova et al., 2015; Kostalova et al., 2016; Martín-Martín et al., 2014; Risueño et al., 2019). It

appears that human anti-*P. perniciosus* antibodies do not recognize rSP03B as strongly as total SGH. Other studies in humans and mice have similarly reported negligible to moderate correlations between SGH and other recombinant proteins such as the anti-clotting protein from *Lutzomyia longipalpis* (Barral et al., 2000) and the D7-protein SP04 from *P. perniciosus* (Martín-Martín et al., 2015). Rohousova et al. (2005) highlighted differences between the intensity of mice' and humans' antibody responses against certain salivary protein in immunoblot assays. Further investigations should be developed to explore the diagnostic use of other recombinant proteins to assess *P. perniciosus* exposure in humans.

Studies in dogs have shown that antibodies against salivary proteins are strongly seasonal, peaking in summer and decreasing thereafter to minimum levels in winter (Kostalova et al., 2015; Vlkova et al., 2011). In experimental trials, anti-*P. perniciosus* IgG antibody levels in dogs, mice and rabbits persisted for at least five weeks, three months, and seven months, respectively (Martín-Martín et al., 2015; Vlkova et al., 2011). In our study LODs to *P. perniciosus* salivary antigens were higher in donors sampled in January-March 2017 compared to those in September-November 2018. Presumably, sand fly exposure was much greater in the summer and autumn 2016 compared to the same time in 2018, as inter-annual variation in sandfly abundance has been reported (Alten et al., 2016). This would also explain the lower TOSV and *L. infantum* prevalence in 2018 than in 2017. Longitudinal serological and entomological studies are needed to characterize the annual antibody dynamics to these infections and to salivary antigens in humans from endemic areas.

LODs for SGH and rSP03B differed with respect to the relationship with demographic variables so it is difficult to stablish solid conclusions concerning risk factors for high levels of *P. perniciosus* salivary gland antibodies. The reason for SGH LODs being higher in donors owning dogs tested for *L. infantum* may reflect the concern of those living in areas of high vector exposure for their dogs becoming infected with the parasite. Higher rSP03B LODs in working dogs may be expected since many probably spend most of their lives outdoors exposed to sand fly bites. Other than possible behavioural reasons leading to greater sand fly exposure there is no apparent biological reason for SGH LODs being marginally higher in women and for rSP03B LODs being inversely associated to age and higher in donors living in flats. Like we found for TOSV positivity, we expected LODs against salivary proteins to be associated to smaller rural areas where sand flies are most abundant. It should be noted however, that none of the areas sampled were heavily

urbanized and all had suitable sand fly breeding environments in the proximity of donor's residences.

The seroprevalence of TOSV in people from Murcia is on the high end of the 2-33% range reported in other studies of Spain, Italy and France (Echevarría et al., 2003; Mendoza-Montero et al., 1998; de Ory et al., 2009), and lower than the 77% reported among forestry workers in some areas in Italy (Valassina et al., 2003). Sand fly vectors are abundant in rural areas in Murcia (Risueño et al., Muñoz) and increasing TOSV seroprevalence with age is compatible with increasing cumulative risk of infection and the same relationship was reported in many other studies (Cardeñosa et al., 2013; de Ory-Manchón et al., 2007; Punda-Polić et al., 2012; Sanbonmatsu-Gámez et al., 2005; Terrosi et al., 2009). The absence of SFSV seropositive individuals agrees with a previous investigation in Murcia (Mendoza-Montero et al. 1998) and contrasts with the 1-5% SFSV seroprevalence reported in other parts of Spain (Mendoza-Montero et al., 1998) and the 1-32% seroprevalence in other Mediterranean countries (Bichaud et al., 2011; Calamusa et al., 2012; Eitrem et al., 1991; Tesh et al., 1976). As previously mentioned, TOSV-associated meningitis has been reported in Murcia on several occasions (Echevarría et al., 2003; Martínez-García et al., 2007; Mendoza-Montero et al., 1998) but given the high seroprevalence, its real clinical impact may be underestimated. TOSV awareness should be raised as this pathogen is a prime candidate in the differential diagnosis of meningitis. Our study indicates that its spatial distribution is highly overdispersed and this is an important consideration from an infection prevention and control perspective.

The study also reflects spatial heterogeneity in the prevalence of *L. infantum* although it did not always coincide with that of TOSV. In a previous study in France TOSV and *L. infantum* infections were positively associated (Bichaud et al., 2011). In our work, six donors (1%) were coinfected with TOSV and *L. infantum*, although both infections were not significantly associated. The epidemiology of these infections is distinctly different. Dogs are the primary reservoir of *L. infantum* but not for TOSV (Muñoz et al., 2020). The epidemiological cycle and reservoir hosts of TOSV have not been determined and possibly infection is maintained between humans and sand fly vectors with no intervention of other hosts required. In any case, it is important to consider that prevalence of *L. infantum* here reported is an underestimation of the true prevalence in Murcia since *L. infantum* typically infects tissue macrophages and most humans do not develop a strong humoral response to infection. A more precise characterization of the parasite's distribution would require

analyzing infection in lymphoid tissue samples and characterizing the specific cellular immune response to the parasite.

Conclusion

Sand fly exposure in southeast Spanish blood donors from periurban and rural areas is widespread and associated to TOSV and *L. infantum* infection. TOSV should be considered an important pathogen in the differential diagnosis of summer meningitis in this part of the country. The SGH provides a reliable marker to assess sand fly exposure instead further studies are required to assess the diagnostic validity of rSP03B and other potential sand fly salivary recombinant proteins.

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Supporting information

Table S1. Median (range) anti-SGH and anti-rSP03B LOD values in blood donors from Murcia Region according to residential variables, occupation and working environment, leishmaniosis awareness, dog's leishmaniosis history, use of insecticides and repellents and *L. infantum* PCR and ELISA results.

Variable	**N**	**Median (range) anti-SGH LOD**	***p* value**	**Median (range) anti-rSP03B LOD**	***p* value**	**N**	**% *L. infantum* positive (PCR+ELISA)**	***p* value**	**N**	**% TOSV seropositive**	***p* value**
Other place of living											
Spain	283	18 (0-67)	0.6789	18 (0-90)	0.1205	234	3.4	0.3304	278	27	0.1059
Abroad	38	18 (0-52)		19 (0-82)		31	6.5		37	14	
Workplace/occupation											
Agriculture	70	15 (0-67)	0.1121	17 (0-56)	0.3953	55	1.8	0.2698	69	33	0.2222
Building sites	32	19 (0-64)		17 (0-64)		28	7.1		32	25	
Factory	82	22 (0-63)		18 (0-62)		71	2.8		82	28	
Hospital	17	23 (0-48)		21 (5-79)		14	0.0		16	19	
Housewife	66	18 (0-64)		19 (0-63)		53	3.8		64	20	
Office	84	22 (0-60)		18 (0-74)		75	9.3		82	21	
Office/outdoors	61	18 (0-53)		20 (0-58)		47	6.4		59	31	
Private homes	26	11 (0-64)		21 (0-82)		22	0.0		26	23	
Professional driver	47	14 (0-51)		16 (0-73)		42	2.4		45	36	
Shop/restaurant	89	16 (0-63)		17 (0-90)		66	3.0		88	33	
Teacher/student	51	19 (0-52)		19 (0-88)		43	0.0		51	14	
Unemployed	29	15 (0-53)		26 (1-52)		22	0.0		29	21	
Outdoors working environment											
Urban only	46	20 (0-57)	0.3002	18 (4-52)	0.1257	39	3.6	0.6487	45	31	0.7266
Includes rural	182	17 (0-67)		16 (0-73)		139	5.1		179	35	

Table S1 (continued). Median (range) anti-SGH and anti-rSP03B LOD values in blood donors from Murcia Region according to residential variables, occupation and working environment, leishmaniosis awareness, dog's leishmaniosis history, use of insecticides and repellents and *L. infantum* PCR and ELISA results.

Variable	N	Median (range) anti-SGH LOD	*p* value	Median (range) anti-rSP03B LOD	*p* value	N	% *L. infantum* positive (PCR+ELISA)	*p* value	N	% TOSV seropositive	*p* value
Leishmaniosis awareness											
Yes	496	18 (0-67)	0.0750	19 (0-90)	0.2293	409	2.9	0.1866	487	27	0.5433
No	171	17 (0-63)		18 (0-88)		139	5.8		169	24	
Leishmaniosis in present and/or previous dogs											
Yes	40	27 (0-67)	0.3684	20 (0-79)	0.1263	31	3.2	1.0000	39	33	0.3031
No	131	22 (0-58)		22 (1-81)		103	4.9		131	24	
Insecticide use											
Frequently	57	6 (0-51)	0.1977	19 (4-90)	0.4577	57	0	1.0000	57	12	1.0000
No/Occasionally	38	7 (0-39)		22 (3-88)		38	0		38	11	
Repellent use in people											
Yes	13	0 (0-51)	0.2184	17 (4-90)	0.9388	13	0	1.0000	13	0	0.1085
No	56	4 (0-34)		18 (3-88)		56	0		56	20	

[1] Only individual PCR results were used for statistical analysis

Table S2. Incidence rate ratios (RRs) from a negative binomial model of the relationship between anti-*P. perniciosus* SGH LODs in blood donors and year, sex, blood donation centre and ownership of dogs being tested or not for *L. infantum.*

Variable	**Level**	**RR**	**95% CI**		***P* value**
Year	2017	1.00	-	-	
	2018	0.29	0.24	0.35	0.0000**
Sex	Male	1.00	-	-	
	Female	1.19	0.99	1.42	0.0625*
Blood donation centre	Almendricos	1.00	-	-	
	Archivel	0.84	0.51	1.40	0.5120
	Barranda	0.91	0.56	1.46	0.6850
	Canteras	2.55	1.63	3.98	0.0000**
	Corvera	2.03	1.34	3.07	0.0008**
	El Paretón	0.63	0.42	0.95	0.0277**
	Fenazar	0.66	0.36	1.19	0.1650
	La Hoya	2.36	1.58	3.53	0.0000**
	La Paca	1.02	0.65	1.62	0.9160
	Librilla	1.30	0.84	2.01	0.2310
	Purias	1.02	0.64	1.64	0.9350
	Sucina	0.68	0.39	1.17	0.1630
	Ulea	1.64	1.10	2.46	0.0160**
Leishmania test	No	1.00	-	-	
	Yes	1.23	1.00	1.51	0.0460**

Table S3. Incidence rate ratios (RRs) from a negative binomial model of the relationship between anti-*P. perniciosus* rSP03B LODs in blood donors and age, year, home type and dog occupation.

Variable	Level	RR	95% CI		*P* value
Age	18-34	1.00	-	-	
	35-50	0.86	0.74	1.00	0.0446**
	51-65	0.80	0.67	0.95	0.0112**
Year	2017	1.00	-	-	
	2018	0.89	0.79	1.01	0.0797*
Blood donation centre	Almendricos	1.00	-	-	
	Archivel	1.08	0.76	1.52	0.6782
	Barranda	1.15	0.83	1.60	0.3897
	Canteras	0.75	0.54	1.04	0.0847*
	Corvera	1.09	0.81	1.47	0.5496
	El Paretón	0.80	0.60	1.05	0.1122
	Fenazar	0.81	0.53	1.25	0.3507
	La Hoya	0.87	0.65	1.16	0.3424
	La Paca	0.64	0.47	0.89	0.0072**
	Librilla	0.82	0.60	1.11	0.2035
	Purias	1.03	0.71	1.48	0.8859
	Sucina	0.82	0.57	1.19	0.3051
	Ulea	1.30	0.98	1.71	0.0687*
Home type	Country house	1.00	-	-	
	Detached	1.64	1.14	2.36	0.0083**
	Flat	1.40	1.04	1.87	0.0261**
	Semi-detached	1.32	1.04	1.68	0.0210**
Dog occupation	Companion	1.00	-	-	
	Work	0.80	0.69	0.93	0.0044**

Table S4. Incidence odds ratios (ORs) from a linear regression model of the relationship between TOSV seropositivity and blood donation centre, year, age, working environment and dog occupation.

Variable	Level	OR	95% CI		*P* value
Blood donation centre	Almendricos	1.00	-	-	
	Archivel	5.93	1.34	26.27	0.0192**
	Barranda	0.44	0.06	3.15	0.4126
	Canteras	1.34	0.30	6.10	0.7025
	Corvera	3.62	0.97	13.55	0.0560*
	El Paretón	2.11	0.58	7.68	0.2597
	Fenazar	0.49	0.07	3.40	0.4697
	La Hoya	0.95	0.24	3.82	0.9478
	La Paca	0.83	0.18	3.79	0.8133
	Librilla	1.89	0.47	7.64	0.3723
	Purias	3.82	0.86	16.91	0.0779*
	Sucina	1.56	0.30	8.01	0.5974
	Ulea	3.72	1.02	13.56	0.0462**
Year	2017	1.00	-	-	
	2018	0.26	0.15	0.48	0.0000**
Age	18-34	1.00	-	-	
	35-50	0.70	0.35	1.41	0.3214
	51-65	2.64	1.21	5.75	0.0144**
Working environment	Outdoors	1.00	-	-	
	Indoors	0.44	0.24	0.80	0.0068**
Dog occupation	Companion	1.00	-	-	
	Work	2.02	1.07	3.81	0.0308**

CHAPTER 4

Genetic diversity and phylogenetic relationships between *Leishmania infantum* from dogs, humans and wildlife in southeast Spain

Abstract

Leishmania infantum causes human and canine leishmaniosis. The parasite, transmitted by phlebotomine sand flies, infects species other than dogs and people, including wildlife, although their role as reservoirs of infection remains unknown for most species. Molecular typing of parasites to investigate genetic variability and evolutionary proximity can help understand transmission cycles and designing control strategies. We investigated *Leishmania* DNA variability in kinetoplast (kDNA) and internal transcribed spacer 2 (ITS2) sequences in asymptomatically infected wildlife (n = 58) and symptomatically and asymptomatically infected humans (n = 38) and dogs (n = 15) from south-east Spain, using single nucleotide polymorphisms (SNPs) and in silico restriction fragment length polymorphism (RFLP) analyses. All ITS2 sequences (n = 76) displayed a 99%–100% nucleotide identity with a *L. infantum* reference sequence, except one with a 98% identity to a reference *Leishmania panamensis* sequence, from an Ecuadorian patient. No heterogeneity was recorded in the 73 *L. infantum* ITS2 sequences except for one SNP in a human parasite sequence. In contrast, kDNA analysis of 44 *L. infantum* sequences revealed 11 SNP genotypes (nucleotide variability up to 4.3%) and four RFLP genotypes including B, F and newly described S and T genotypes. Genotype frequency was significantly greater in symptomatic compared to asymptomatic individuals. Both methods similarly grouped parasites as predominantly or exclusively found in humans, in dogs, in wildlife or in all three of them. Accordingly, the phylogenetic analysis of kDNA sequences revealed three main clusters, two as a paraphyletic human parasites clade and a third including dogs, people and wildlife parasites. Results suggest that *Leishmania infantum* genetics is complex even in small geographical areas and that, probably, several independent transmission cycles take place simultaneously including some connecting animals and humans. Investigating these transmission networks may be useful in understanding the transmission dynamics, infection risk and therefore in planning *L. infantum* control strategies.

Introduction

Leishmania are kinetoplastid protozoa that include up to 54 different species that are endemic in at least 98 countries (Akhoundi et al., 2017). *Leishmania infantum* is one of the 20 species pathogenic to humans (Akhoundi et al., 2016) and is responsible for life-

threatening human leishmaniosis (HumL) and canine leishmaniosis (CanL). The parasite is transmitted to vertebrate hosts by bites of infected female phlebotomine sand flies, being *Phlebotomus perniciosus* and *P. ariasi* its vector species in Western Europe (Campino et al., 2006; Martin-Sanchez, Gramiccia, Di Muccio, Ludovisi, & Morillas-Márquez, 2004). Whilst the dog is the main reservoir host of the parasite in the domestic environment, humans are considered an accidental host (Otranto et al., 2015; Quinnell & Courtenay, 2009). Moreover, other mammals, including a wide range of wildlife, have been molecularly diagnosed as *L. infantum*-positive (Del Río et al., 2014; Millán, Ferroglio, & Solano-Gallego, 2014; Risueño et al., 2018). However, clinical signs are rarely reported in wildlife and most infected individuals have low parasite loads. Moreover, their ability to infect sand flies has been proven by xenodiagnoses only in a few species (Jiménez, González, Martín-Martín, Hernández, & Molina, 2014; Molina et al., 2012; Quinnell & Courtenay, 2009). Consequently, the reservoir role of most species in which *L. infantum* infection has been detected remains unknown, limiting our understanding of the epidemiology of the infection (Millán et al., 2011; Silva, Gontijo, & Melo, 2005). Further insight into the nature of domestic and sylvatic transmission cycles and the interaction with each other may be obtained by comparing *L. infantum* infecting different hosts (Cortes et al., 2006). It would be expected that hosts living close to each other and harbouring the same strains are exposed to the same infected sand flies, suggesting a common epidemiological cycle.

According to the gene target, variability in DNA sequences may reflect evolutionary divergence and can be used to elaborate phylogenetic hypothesis. A wide range of DNA targets have been used to study genetic variability and phylogeny within *Leishmania* genus (Akhoundi et al., 2017). Among them, the mini exon/spliced leader gene (Azmi, Nasereddin, Ereqat, Schönian, & Abdeen, 2010; Marfurt, Niederwieser, Makia, Beck, & Felger, 2003), the hsp70 gene (Montalvo, Fraga, Maes, Dujardin, & Van Der Auwera, 2012), the ribosomal internal transcribed spacer (ITS1-2) regions (de Almeida et al., 2011; Del Río et al., 2014; Schönian et al., 2003) and the kinetoplast, mitochondrial DNA (kDNA) (Millán et al., 2011; Souza Castro et al., 2018) have enough sequence polymorphisms to allow sensitive detection of genetic variability within *Leishmania* species (Akhoundi et al., 2017). Their high copy number and the presence of variable and conserved DNA regions make them ideal for pathogen detection (high sensitivity) and typing (variability studies) (de Almeida et al., 2011; Schönian et al., 2003; Silva, Richini-

Pereira, Kikuti, Marson, & Langoni, 2017). Intraspecific variability was recorded in the internal transcribed spacer 2 (ITS2) sequence of *L. infantum* from humans, dogs and wildlife from Spain (Del Río et al., 2014). This sequence is 50–650 bps long (Akhoundi et al., 2017), and the number of copies of the ribosomal gene is ~160 (Leon, Fouts, & Manning, 1978). kDNA is particularly suited for investigations in asymptomatically *L. infantum* infected individuals with a very low parasite burden (Pérez-Cutillas et al., 2015), as 95% of its DNA is arranged into characteristic minicircles consisting of thousands of copies/cell of a ~0.8 kb sequence of which ~120 bps are well conserved and the rest of the sequence is variable between and within *Leishmania* species (Kocher, Valière, Bañuls, & Murienne, 2017).

Restriction fragment length polymorphism preceded by polymerase chain reaction (PCR-RFLP) on various gene targets, including kDNA, has been widely used for *Leishmania* typing (Chicharro et al., 2002; Cortes et al., 2006; Salvatore et al., 2016; Sobrino et al., 2008), and to date, 18 different RFLP genotypes named from A to R have been characterized by this method (Cortes et al., 2006; da Silva et al., 2015; El Hamouchi, Ejghal, Hida, & Lemrani, 2017). The above genotypes have been also related with the human patients' immunological status, with genotypes A and B being more commonly found in immunocompromised and immunocompetent individuals, respectively (Cortes et al., 2006). In a recent study, Risueño et al. (2018) assigned *L. infantum* from seven foxes from Murcia Region in south-east Spain, to RFLP genotype B, which is the most frequent in humans from Spain, Brazil and Morocco and in dogs from the latter two countries. The detection in this study of common genotypes among parasites from dogs, humans and wildlife, coupled with spatially matching CanL and HumL and vector distributions, suggested a common epidemiological cycle of infection.

The present study extended the characterization of *L. infantum* genetic variants from south-east Spain to other symptomatically and asymptomatically infected wildlife, dogs and humans using in silico kDNA PCR-RFLP and single nucleotide polymorphism (SNP) analysis of kDNA and ITS2 sequences. The objective was to improve our understanding of domestic and sylvatic transmission cycles of *L. infantum* in a typical yet relatively small geographical area and investigate potential associations between parasite genotypes and clinical and epidemiological traits.

Materials and methods

Study design and samples

The study included one hundred and eleven DNA samples from humans (n = 38), dogs (n = 15) and wildlife (n = 58) diagnosed *L. infantum*-positive by a real-time PCR (rt-PCR) assay (Dantas-Torres, da Silva Sales, Gomes da Silva, Otranto, & Figueredo, 2017; Francino et al., 2006). They came from the endemic Region of Murcia and the neighbouring province of Alicante in south-east Spain and had been taken between 2008 and 2017 (Figure 1).

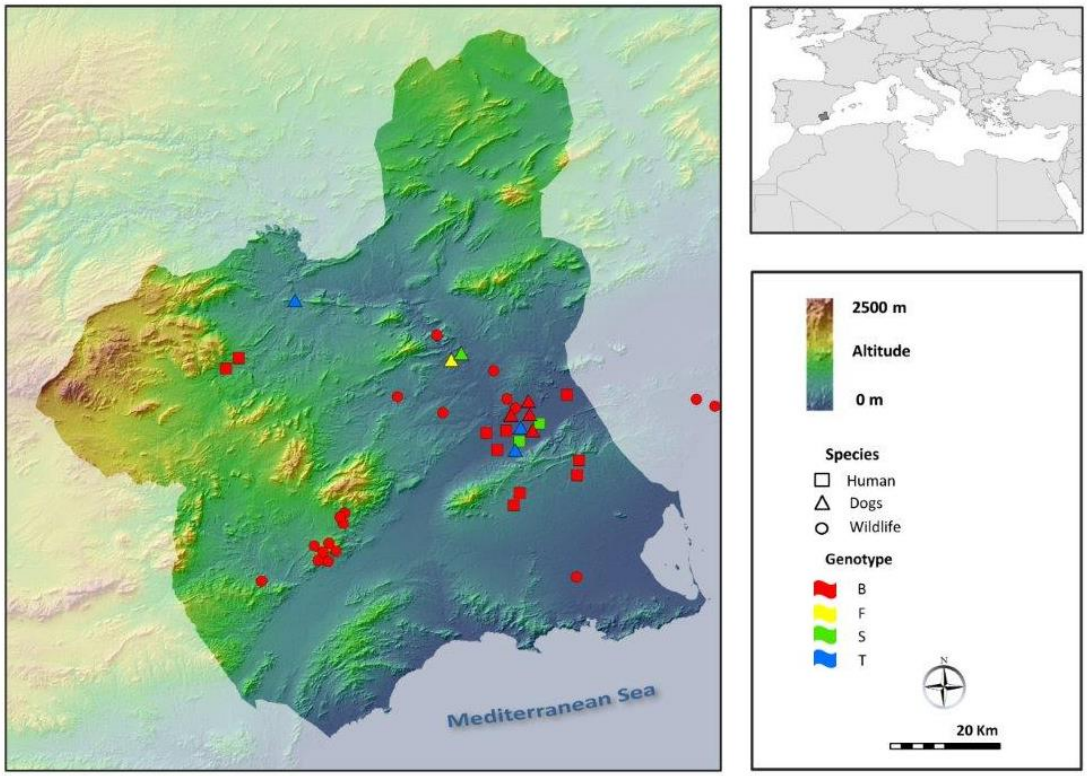

Figure 1. Spatial distribution of *Leishmania infantum* kinetoplast DNA-restriction fragment length polymorphism genotypes from humans, dogs and wildlife in Murcia in south-east Spain.

Human DNA samples were obtained from bone marrow, blood and skin from 33 clinical cases, 24 with human visceral leishmaniosis (HVL) and nine with human cutaneous leishmaniosis (HCL) and blood from five asymptomatically infected blood-donors. HVL cases included four human immunodeficiency virus (HIV)-positive patients. HVL and HCL samples were obtained in La Arrixaca University Clinical Hospital in Murcia City, and those from blood donors were collected from around the region by Murcia Blood

Donation Centre. Dog samples comprised DNA from 15 *L. infantum* culture isolates (14 from spleen and one from lymph node) from 12 individuals of which seven were asymptomatic, four had CanL symptoms, and one had an unknown clinical status. They were abandoned dogs, euthanized at the Zoonosis Control Centre in Murcia. Wildlife samples were all from asymptomatic animals and were obtained from spleen, liver or skin samples from 26 foxes (*Vulpes vulpes*), 17 wild rabbits (*Oryctolagus cuniculus*), five brown rats (*Rattus norvegicus*), three beech martens (*Martes foina*), two wolves (*Canis lupus*), two wild mice (*Apodemus sylvaticus*), one genet (*Genetta genetta*), one brown bear (*Ursus arctos*) and one wild cat (*Felis silvestris*). Rabbits and foxes had been legally hunted, and other animals were found dead mostly following road traffic accidents, by local authorities, except for the brown bear and the wolves which came from a zoological park in Murcia. Skin punch biopsies were obtained from the latter when anaesthetized for unrelated reasons. One of the foxes included in this study had been previously amplified and RFLP-classified as genotype B by Risueño et al. (2018).

DNA extraction and PCR amplification of target sequences

DNA had been extracted from samples using an automatized system (Maxwell® Promega) and concentration and purity analysed using NanoDrop 2000® spectrophotometer (Thermo Fischer Scientific). Positive rtPCR samples were amplified by conventional, endpoint PCRs (cPCR) using primers targeting a 447-bp fragment of the minicircle kDNA (Cortes, Rolão, Ramada, & Campino, 2004) and a 418-bp fragment of the ITS2 region of *L. infantum* (de Almeida et al., 2011). The forward minicircle primer (MC1, 5′-GTTAGCCGATGGTGGTCTTG-3′) is situated in the variable region instead, the reverse primer (MC2, 5′-CACCCATTTTTCCGATTTTG-3′) is located in the conserved region, and the amplified fragment includes 405 bp from the variable region and 42 bp from the conserved region (Cortes, 2008). The 418bp-ITS2 fragment is found in a variable region, including primers LGITSF2 (5′-GCATGCCATATTCTCAGTGTC-3′) and LGITSR2 (5′-GGCCAACGCGAAGTTGAATTC-3′) (de Almeida et al., 2011).

Polymerase chain reactions consisted of 2 µl of DNA and 23 µl of PCR mix containing 2.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl, 250 µM of each dNTP, 50 pmol of each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems®). The thermocycling profiles for kDNA/ITS2, respectively, were one cycle at 94/95°C for 10 min followed by 36 cycles at 94/95°C for 30 s, 60/64°C for 30 s and 72°C for 30 s/1 min, and

a final step at 72°C for 5/7 min. DNA from bone marrow from a L. *infantum*-infected dog and a DNA-free water sample were used as positive and negative controls. Amplified products were subjected to electrophoresis in 2% agarose gel stained with GelRed (VWR International PBI) and visualized on a Gel Logic 100 Imaging System® (Kodak).

Sequencing and single nucleotide polymorphism (SNPs) analysis

Amplified products of ITS2 and kDNA were purified enzymatically with phosphatase and exonuclease enzymes (ThermoFisher Scientific) and sequenced in both directions with the same primers used in the previous PCRs, using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fischer Scientific) in a 3130 Genetic Analyzer (Applied Biosystems). Sequences were compared with those available in GenBank database, using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Sequences with the highest (100%) query cover and identity for most sequences were selected as reference strains (KX098509 for kDNA; KU680960 for ITS2) for comparison following sequence alignment with the ClustalW program (Larkin et al., 2007) and the BioEdit software (Alzohairy, 2011).

Genetic distance (expressed in %) among representative sequences identified, was calculated using the Kimura 2-parameter substitution model with gamma distributed (G) rates (Kimura, 1980), implemented in the MEGA 7 software (Kumar, Stecher, & Tamura, 2016).

Restriction fragment length polymorphism (RFLP) analysis

kDNA sequence variability was also investigated by in silico RFLP analysis with the RestrictionMapper online program (http://www.restrictionmapper.org/). A panel of nine endonucleases (*Bgl*II, *Bme1390*I, *Dde*I, *Hpa*II, *Rsa*I, *Vsp*I, *Pst*I, *Sfc*I, *Xap*I) was used, and according to the number and size of the fragments obtained, sequences were classified into the RFLP genotypes described by Cortes et al. (2006) and El Hamouchi et al. (2017).

Phylogenetic analyses

The phylogenetic relationships between genetic variants were inferred by neighbor-joining (NJ) method and p-distance model using MEGA7 software (Kimura, 1980; Kumar et al., 2016). Homologous sequence of *Trypanosoma avium* (Accession number

AF027214) and of *Trypanosoma evansi* (LC199491) were used as outgroup sequences for kDNA and ITS2, respectively.

A matrix scoring the presence or absence of each band in each genotype was built up and a maximum-likelihood (ML) tree was produced using RESTML (PHYLIP package, version 3.695, available at http://evolution.genetics.washington.edu/phylip.html).

Data mapping and statistical analysis

The geographical location of human's residences and those where wildlife and dogs were collected were georeferenced. They were used to create a map and to analyse the spatial autocorrelation of *L. infantum* genotypes using Global Moran's I Index, in ArcGIS v.10 (ESRI) geographical information system. The proportion of genotypes according to levels of explanatory variables including host species, gender, age, presence of symptoms and sample type and season of collection was calculated and compared using Fisher's exact test, using R software (http://cran.r-project.org/). Differences were considered statistically significant for $p<0.05$ for a two-tailed test.

Results

Frequency of amplified and sequenced kDNA and ITS2 regions

All dog samples were cPCR-positive in both kDNA and ITS2 regions, whilst the percentage of positive human, fox and rabbit samples ranged between 59% and 79% for the ITS2 sequence and 16%–47% for kDNA ($p < .05$). No amplifications were obtained from wild cat samples (Table 1). Good-quality sequence information was obtained from 44 kDNA and 73 ITS2 amplicons (Table 1).

Single nucleotide polymorphism (SNP) analysis

BLAST analysis of ITS2 sequences indicated that all samples scored positive to *L. infantum* (99%–100% identity with the reference sequence KU680960) except one sample from a HCL, HIV-negative, Ecuadorian patient which was positive for *Leishmania panamensis* (98% identity with GenBank FJ948445). Among all *L. infantum* ITS2 sequences, two types were identified namely as type I (n = 71) identical to the reference

sequence, and type II (n = 1) showing one SNP (G insertion at 326_327 position) in a human parasite sequence.

Table 1. Frequency of *L. infantum* PCR amplified and sequenced samples according to host.

Host	No.	No. (%) PCR-positives		No. (%) sequences	
	Samples	kDNA	ITS2	kDNA	ITS2
Humans	38	14 (37)	30 (79)	11 (29)	26 (68)
Dogs	15	15 (100)	15 (100)	14 (93)	15 (100)
Wildlife	58	21 (36)	41 (71)	19 (33)	32 (55)
Fox	26	5 (19)	19 (73)	4 (15)	15 (58)
Rabbit	17	8 (47)	10 (59)	8 (47)	9 (53)
Rat	5	2 (40)	5 (100)	1 (20)	2 (40)
Beech marten	3	3 (100)	3 (100)	3 (100)	3 (100)
Wolf	2	1 (50)	1 (50)	1 (50)	1 (50)
Mouse	2	0 (0)	1 (50)	0 (0)	0 (0)
Genet	1	1 (100)	1 (100)	1 (100)	1 (100)
Brown bear	1	1 (100)	1 (100)	1(100)	1 (100)
Wild cat	1	0 (0)	0 (0)	0 (0)	0 (0)
TOTAL	111	50 (45)	86 (77)	44 (40)	73 (66)

BLAST analysis of kDNA identified all positive samples as *L. infantum* (kDNA was not available from the *L. panamensis* sample) with sequence identity ranging 97%–100% when compared with the reference strain (KX098509), and 1–14 SNPs were detected in 29 of the 44 kDNA sequences. Sequences were grouped into 11 different SNP genotypes (namely G1-11, Table 2) and the percentage of nucleotide variability ranged from 0% to 4.3% (Table 3). *Leishmania infantum* genotype distribution according to the host species is reported in Table 2. Genotype 1 was found in dogs, humans and wildlife; genotype 2 in dogs and wildlife; genotypes 3, 4 and 5 only in dogs; genotype 6 in humans and dogs; and SNP genotypes 7, 8, 9, 10 and 11 were present only in humans. Spleen and popliteal lymph node samples from one dog presented parasites belonging to different SNP genotypes (G1 and G5, respectively). Overall, the frequency of SNP types identified included 55 substitutions (48 transitions and seven transversions), two deletions and three insertions (Table 2). The complete alignment of 447 bp kDNA sequences is provided as Table S1.

Table 2. Frequency and characteristics of kDNA SNP-derived genotypes according to host and human clinical form and relationship with RFLP-genotypes.

	Variable nucleotide sites identified within the kDNA sequences[a]																															
SNP-genotype	**6**	**51**	**59**	**67**	**102**	**105**	**152**	**164**	**167**	**174**	**175**	**185**	**195**	**211**	**259**	**296**	**309**	**317**	**318**	**319**	**334**	**336**	**343**	**345**	**353**	**364**	**367**	**No. of SNPs**	**No. dogs**	**No. wildlife**	**No. humans**	**RFLP-genotype**
KX098509	A	C	T	A	C	A	A	A	T	T	A	T	A	A	A	G	-	G	A	A	T	G	A	G	G	G	G					
1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	-	·	·	·	·	·	·	·	·	·	·	0	9	1[b]	5	B
2	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	-	·	·	·	·	·	·	·	·	·	·	1	1	18[c]	0	B
3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	-	T	·	·	·	·	·	·	·	·	·	1	1	0	0	B
4	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	-	·	·	·	·	·	·	·	·	·	·	1	1	0	0	S
5	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	-	·	·	G	·	·	·	·	·	·	·	2	1	0	0	B
6	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	-	·	·	·	·	·	·	·	·	A	·	3	1	0	1	S
7	G	·	·	·	A	·	G	·	·	·	·	C	·	·	·	·	-	·	·	·	C	·	·	·	·	·	·	5	0	0	1	F
8	G	·	·	·	A	·	G	·	·	·	·	·	·	G	·	-	·	G	·	C	·	T	·	·	·	·	·	7	0	0	1	F
9	G	·	C	·	A	G	·	·	·	·	G	·	·	G	·	A	T	·	·	·	C	A	·	A	A	·	A	13	0	0	1	T
10	G	-	C	·	A	G	·	·	·	·	G	·	·	·	·	A	T	·	·	·	C	A	·	A	A	·	A	13	0	0	1	T
11	G	·	C	·	A	G	·	·	C	·	G	·	G	·	·	A	T	·	·	·	C	A	·	A	A	·	A	14	0	0	1	T

[a] Alignment of the kDNA *Leishmania* sequences. Nucleotide identity in each position in relation to the reference sequence KX098509 is indicated with a dot (·), indels are indicated with a dash (-).

[b] Rabbit.

[c] Brown bear (1), beech marten (3), rabbit (7), fox (4), genet (1), wolf (1), rat (1).

Table 3. Estimates of evolutionary divergence (%) between SNP-genotypes using the Kimura 2-parameter model.

	G3	G1	G2	G4	G5	G6	G7	G8	G10	G9	G11
G3											
G1	0.3										
G2	0.3	0.0									
G4	0.5	0.3	0.3								
G5	0.8	0.5	0.5	0.8							
G6	1.0	0.8	0.8	0.5	1.3						
G7	1.5	1.3	1.3	1.5	1.8	2.0					
G8	2.0	1.8	1.8	2.0	2.3	2.6	1.0				
G10	3.1	2.9	2.9	3.1	3.4	3.7	2.6	3.1			
G9	3.4	3.1	3.1	3.4	3.7	4.0	2.9	3.4	0.3		
G11	3.7	3.4	3.4	3.7	4.0	4.3	3.1	3.7	0.5	0.8	

In silico RFLP genotyping

Restriction fragment length polymorphism analysis of kDNA sequences revealed four RFLP genotypes, including previously described B and F (Cortes et al., 2006) and two new ones, named RFLP genotypes S and T. The latter were defined based on a new restriction pattern obtained for *Rsa*I (pattern III) combined with *Hpa*II patterns I and II, respectively (Tables 4 and 5). Genotype B, present in parasites from dogs, humans and all wildlife species, was the most frequent type (82%) herein retrieved, followed by genotypes F (5%) and T (7%) from human parasites and S (7%) from parasites isolated from a human and dogs (Table 2). Comparing RFLP genotypes with the SNP analysis, genotype B encompasses four SNP variants (1, 2, 3 and 5), genotype F and S include two SNP variants each (7 and 8, and 4 and 6, respectively), whilst genotype T includes three SNP variants (9, 10 and 11). The two SNP genotypes detected in parasites from spleen and lymph node from the same dog belonged to genotype B.

Table 4. Enzymatic digestion patterns with the corresponding fragment size.

Enzymes	***Bgl*II**		***Bme1390*I**				***Dde*I**			***Hpa*II**					***Rsa*I**				***Vsp*I**			***Pst*I**	***Sfc*I**	***Xap*I**	
Patterns	**I**	**II**	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**I**	**II**	**III**	**IV**	**V**	**I**	**II**	**III[a]**	**IV**	**I**	**II**	**III**	**I**	**I**	**I**	**II**
Fragments (bp)	447	258	411	288	447	324	319	419	240	410	287	447	350	324	253	210	447	253	161	170	310	298	224	347	347
		189	36	123		123	100	28	180	37	123		57	123	194	197		144	150	130	137	149	153	60	100
				36			28		28		37		37			40		48	136	90			70	40	
																				50					

[a] New pattern discovered in this study for *Rsa*I enzyme, named III, as this name for *Rsa*I enzyme has not been previously used

Table 5. RFLP-genotypes described and corresponding enzyme patterns in *L. infantum* from dogs, humans and wildlife hosts.

Genotypes	*Bgl*II	*Bme1390*I	*Dde*I	*Hpa*II	*Rsa*I	*Vsp*I	*Pst*I	*Sfc*I	*Xap*I
A	II	I	II	I	I	I	I	I	I
B	I	I	I	I	I	I	I	I	I
C	II	I	III	I	I	I	I	I	I
D	I	II	I	II	I	I	I	I	I
E	I	I	II	I	I	I	I	I	I
F	I	I	I	IV	I	I	I	I	I
G	I	I	I	I	II	II	I	I	I
H	II	I	I	I	II	II	I	I	I
I	II	I	I	I	I	II	I	I	I
J	I	I	I	I	IV	I	I	I	I
K	I	III	I	III	I	I	I	I	I
L	I	II	I	II	IV	I	I	I	I
M	I	III	I	IV	II	I	I	I	I
N	I	I	I	III	I	I	I	I	I
O	I	I	I	I	I	II	I	I	I
P	I	I	I	I	I	III	I	I	I
Q	I	I	I	I	II	II	I	I	II
R	I	IV	I	V	I	I	I	I	I
S[a]	I	I	I	I	III[b]	I	I	I	I
T[a]	I	I	I	II	III[b]	I	I	I	I

[a] New RFLP-genotypes

[b] New pattern discovered in this study

Phylogenetic analyses

The 11 kDNA SNP genotypes clustered into three different groups according to the NJ tree (Figure 2). Two of these independent clusters were composed by human-specific variants (F and T RFLP genotypes, corresponding to SNP genotypes 7–8 and 9–11, respectively), whereas dog-specific and host-shared variants grouped together (B and S RFLP genotypes, corresponding to SNP genotypes 1–6). This clustering coincides with that of the ML phylogenetic tree from RFLP data of all RFLP genotypes described in the literature so far (A–T; Figure 3), with B and S genotypes clustering together and separated from F and T, with each one in an independent cluster.

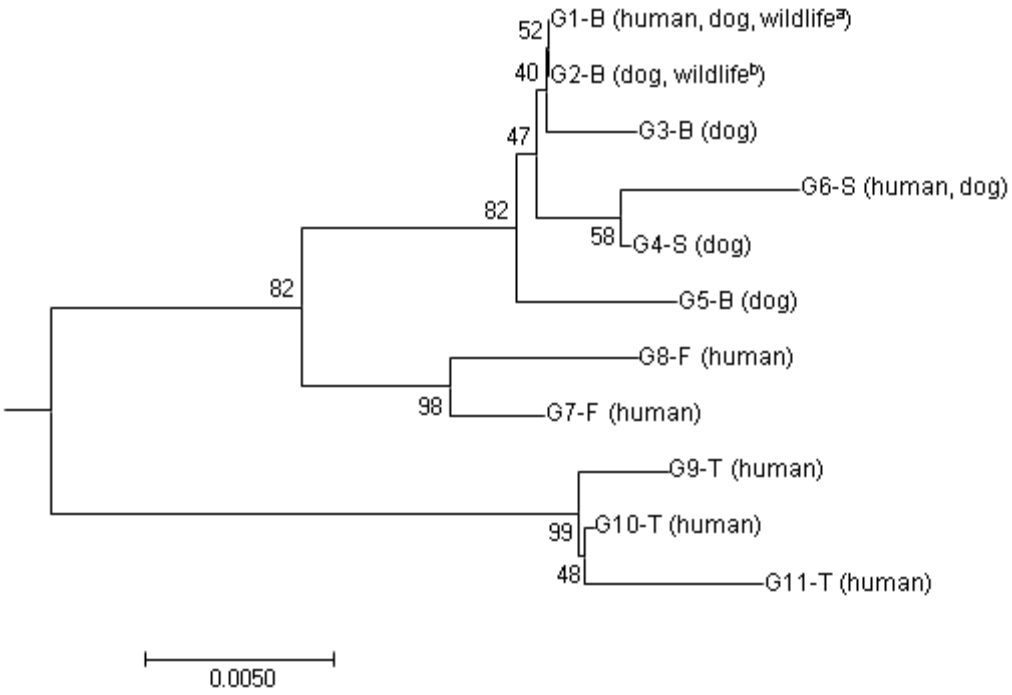

Figure 2. Neighbor-joining phylogenetic tree of *Leishmania infantum* from humans and animals in south-east Spain. The outgroup branch is not shown and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (8,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. [a]Rabbit, [b]Brown bear (1), beech marten (3), rabbit (7), fox (4), genet (1), wolf (1), rat (1).

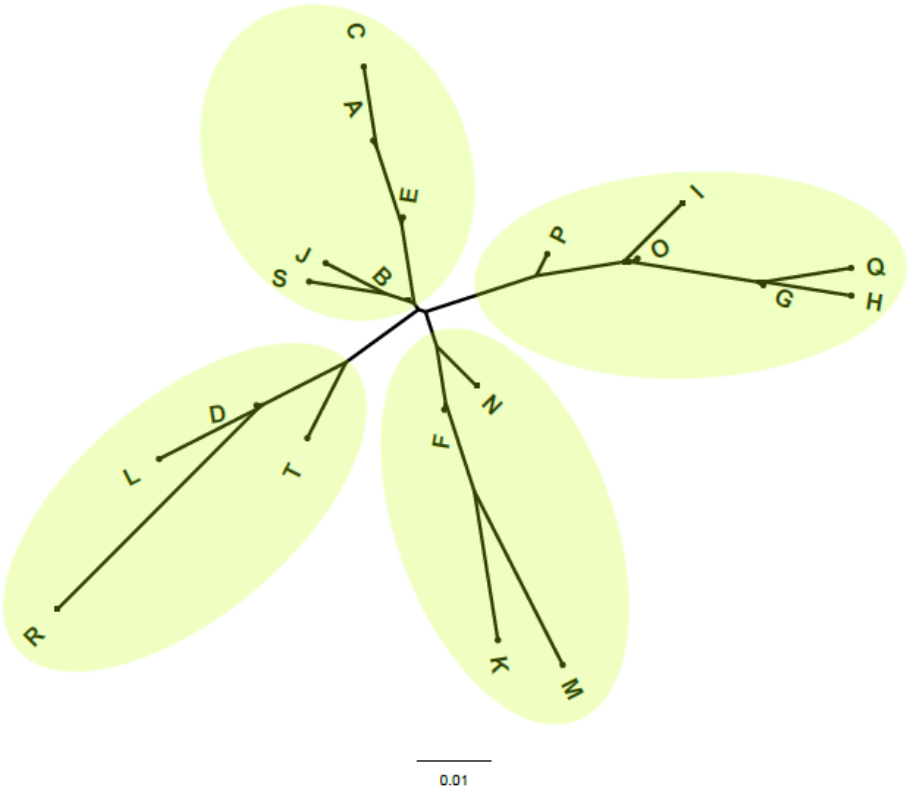

Figure 3. Maximum-likelihood phylogenetic tree from restriction sites derived from all existing kinetoplast DNA polymerase chain reaction-restriction fragment length polymorphism *Leishmania infantum* genotypes described so far in the literature.

Epidemiological and clinical features associated with the genotypes

Information concerning the 44 kDNA sequences obtained (including host tissue, demographic and clinical data, as well as resulting RFLP and SNP genotypes) is shown in Table S2 in the supplementary file. As shown in Table 6, *L. infantum* RFLP and SNP genotype distributions varied according to host species and the presence or absence of symptoms. In contrast to symptomatic individuals (dogs and humans), genomes for most asymptomatically infected individuals were restricted to RFLP genotype B and SNP genotypes 1 and 2. However, there was no association between specific genotypes and the clinical presentation in human patients (Table 6). Moreover, a kDNA sequence was not obtained from parasites belonging to the four HIV-positive patients. Additionally, SNP genotype frequency differed according to the tissue sample. Finally, there was no evidence of spatial autocorrelation among *L. infantum* genotypes ($p<0.05$; Figure 1).

Table 6. Percentage of individuals with specific *L. infantum* genotypes according to study independent variables.

	No. of samples	% Restriction fragment length polymorphism genotypes					% SNP-genotypes											
		B	F	S	T	*p*-value	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	*p*-value
Host																		
Human	11	45	18	9	27	0.0002	45	0	0	0	0	9	9	9	9	9	9	<0.0001
Dog	14	86	0	14	0		64	7	7	7	7	7	0	0	0	0	0	
Wildlife	19	100	0	0	0		5	95	0	0	0	0	0	0	0	0	0	
Sex																		
Female	22	77	5	5	14	0.4362	23	45	5	0	5	5	5	0	5	5	5	0.6619
Male	20	85	5	10	0		45	40	0	5	0	5	0	5	0	0	0	
Age																		
Young	4	75	0	0	25	0.5582	25	50	0	0	0	0	0	0	0	0	25	0.2466
Adult	23	83	9	4	4		48	35	0	0	0	4	4	4	0	4	0	
Senior	4	75	0	0	25		25	25	0	0	25	0	0	0	25	0	0	
Presence of symptoms																		
Symptomatic	18	61	11	11	17	0.0072	56	0	0	0	6	11	6	6	6	6	6	<0.0001
Asymptomatic	25	96	0	4	0		16	76	4	4	0	0	0	0	0	0	0	
Human clinical form																		
HCL[a]	2	0	50	0	50	0.3636	0	0	0	0	0	0	50	0	0	50	0	0.2727
HVL[b]	9	56	11	11	22		56	0	0	0	0	11	0	11	11	0	11	
Tissue																		
Spleen	20	90	0	10	0	0.0977	45	40	5	5	0	5	0	0	0	0	0	<0.0001
Skin	11	82	9	0	9		0	83	0	0	0	0	8	0	0	8	0	
Bone marrow	8	63	0	13	25		63	0	0	0	0	13	0	0	13	0	13	
Liver	2	100	0	0	0		50	50	0	0	0	0	0	0	0	0	0	
Lymph node	1	100	0	0	0		0	0	0	0	100	0	0	0	0	0	0	
Blood	1	0	100	0	0		0	0	0	0	0	0	0	100	0	0	0	
Season																		
Spring	10	70	10	10	10	0.9548	50	20	0	0	0	10	0	10	0	0	10	0.3390
Summer	3	100	0	0	0		0	100	0	0	0	0	0	0	0	0	0	
Autumn	8	75	13	0	13		50	13	0	0	13	0	13	0	13	0	0	
Winter	14	79	0	14	7		36	43	0	7	0	7	0	0	0	7	0	

[a] Human cutaneous leishmaniosis
[b] Human visceral leishmaniosis

Discussion

Study findings suggest wide genetic variability in *L. infantum* from a relatively small geographical region in south-east Spain. The understanding of such a genetic variability of *L. infantum* amongst different host species is relevant from an epidemiological and clinical perspective. In contrast to kDNA, ITS2 sequences from *L. infantum* were highly homogeneous but clearly different to those of *L. panamensis*, supporting its adequacy for *Leishmania* species identification (Akhoundi et al., 2017). The later came from an Ecuadorian HCL patient, presumably infected in Latin-America which is the only region of the world where this species is endemic (van der Auwera & Dujardin, 2015; Marsella & de Gopegui, 1998). The homogeneity in the ITS2 compared to the variability in kDNA sequences could explain the greater success in amplifying the former region using conventional PCR. The high polymorphism in kDNA minicircles reflects its fast evolutionary rate (Akhoundi et al., 2017; Noyes, Reyburn, Bailey, & Smith, 1998) and is epidemiologically useful for identifying low geographical-scale transmission cycles. Hosts from a small geographical zone bearing the same *L. infantum* SNP genotypes are likely to be part of a common transmission cycle. The epidemiological relevance of similar SNP variants such as genotypes 2, 3 and 4 differing in one SNP only is not evident, and mutations in kDNA were described following long-term in vitro culture of the parasite (Cortes et al., 2006). Moreover, individual *Leishmania* parasites contain several different minicircle sequences (classes), some of them differing in a small number of bases (Brewster & Barker, 2002; Kocher et al., 2017). Phylogenetic analysis supported three main sequence groups, one clustering human, dog and wildlife parasites and two grouping human parasites variants containing up to 14 SNPs compared to the reference sequence. Greater heterogeneity is possibly related to broader exposure of people to different *L. infantum* variants from wider travelling and to immunological differences between hosts (Alvar et al., 1997).

Restriction fragment length polymorphism was less discriminating than SNP genotyping, but it was similarly able to group *L. infantum* variants specific to humans (genotypes T and F), humans and dogs (genotype S) and those present in humans, dogs and wildlife (genotype B). *Leishmania infantum* genotypes B and F appear to be widespread in humans in Europe and Latin America (Cortes et al., 2006; da Silva et al., 2015; Risueño et

al., 2018; Schönian et al., 1996). The former was also found in foxes in Murcia (Risueño et al., 2018) but herein described for the first time in dogs from Spain. Genotype F was detected here among HVL and HCL patients of similar age (adults) and different nationalities (Spanish and non-Spanish), and also in two immunocompromised HVL Portuguese patients, one HCL case in France (Cortes et al., 2006 from Blaineau, Bastien, & Pagès, 1992; Cortes et al., 2006) and in one *Leishmania chagasi* (syn. *L. infantum* HCL patient from Panama (da Silva et al., 2015 from Schönian et al., 1996)). The presence of the same genotypes (B and F) in both continents would be expected since *L. infantum* was introduced in America following the arrival of the Iberian colonizers (Kuhls et al., 2011; Lukes et al., 2007). In contrast, genotypes S and T have not been reported elsewhere.

Together, these findings indicate that small geographical areas have a variety of *L. infantum* genetic variants including local ones and others described in several countries, which circulate predominantly within or between host species. Although *L. infantum* variants have been previously found in dogs or humans only (Alvarenga et al., 2012; Cortes et al., 2006), there are no reports of clear host-specificity of *L. infantum* strains, and sand fly vectors feed indiscriminately from a wide range of mammal and avian host (Bongiorno, Habluetzel, Khoury, & Maroli, 2003; Maia et al., 2013). In contrast, certain *L. infantum* isoenzymatic variants (zymodemes) have been associated to particular epidemiological situations such as those circulating among human intravenous drug users in Spain (Alvar et al., 1997).

High polymorphism among Spanish *L. infantum* was also reported when using other typing techniques such as multilocus enzyme electrophoresis (Martin-Sanchez et al., 2004). Strain comparisons within and between countries are, however, complicated by the large number of methods and sequences used, and there is a clear need for unification and standardization of molecular markers for a better understanding of *Leishmania* epidemiology (Akhoundi et al., 2017; van der Auwera & Dujardin, 2015; Schönian, Kuhls, & Mauricio, 2010). There is little evidence so far for a strong association between *L. infantum* infection in humans with specific variants and certain clinical forms, partly because the tropism of this species for the skin or for multiple internal organs (i.e., cutaneous/visceral forms) is mostly influenced by the host immune response (Alvar, 1994; Gradoni & Gramiccia, 1994; Rossi & Fasel, 2018). This was the case in this study where genotypes did not relate to human disease presentations. Notwithstanding this, genotype frequency differed in symptomatic and asymptomatic individuals, and according to the type

of sample analysed. This is likely to be related to the strong correlation between the presence/absence of symptoms, host species, genotype frequency and the sample type analysed; for example, all human parasites genotypes were from symptomatic patients and most were tested in bone marrow samples only. Instead, all wild animals were asymptomatic and analysed in spleen or skin samples only. Clearly, there is a need to further investigate the relationship between *L. infantum* genetic variants and clinical presentations and tissue tropism, employing a larger number of samples. An issue that can further complicate the relationship between parasite strains and the clinical outcome is the possibility of simultaneous coinfections with different parasite variants. Two different SNP genotypes (1 and 5) were found in parasites from spleen and lymph nodes of one dog, which could imply that it was infected with two *L. infantum* variants or, alternatively, that the same variant contained several predominant minicircle classes (Brewster & Barker, 2002) readily amplified with the PCR protocol used.

In summary, the present study provides some evidence of the genetic complexity of *L. infantum* populations in small geographical areas, with a large number of genotypes circulating in the domestic and sylvatic environment, some predominantly found in human, dog or wildlife parasites and other shared by all three. This suggests that a number of independent and common parasite transmission cycles exist, and further detailed investigation of transmission networks could help improve *L. infantum* control. This should include studies on parasites isolated from sand fly vectors, and using additional molecular markers.

Conclusions

A wide range of genetic *L. infantum* variants is present in small populations that cluster among certain host species or shared by humans and domestic and wild animals. Most likely, this is the result of different independent and interconnecting parasite transmission cycles.

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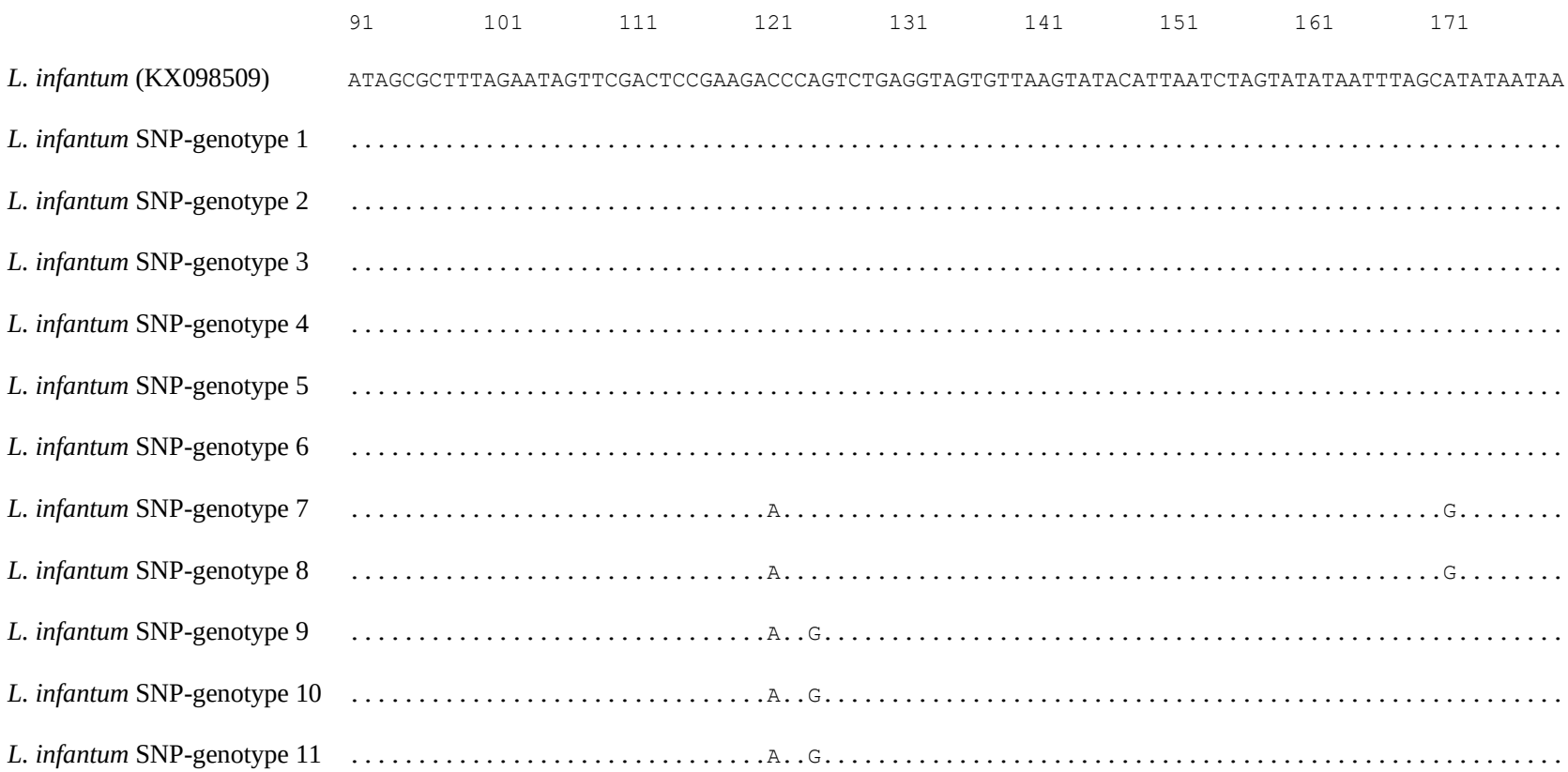
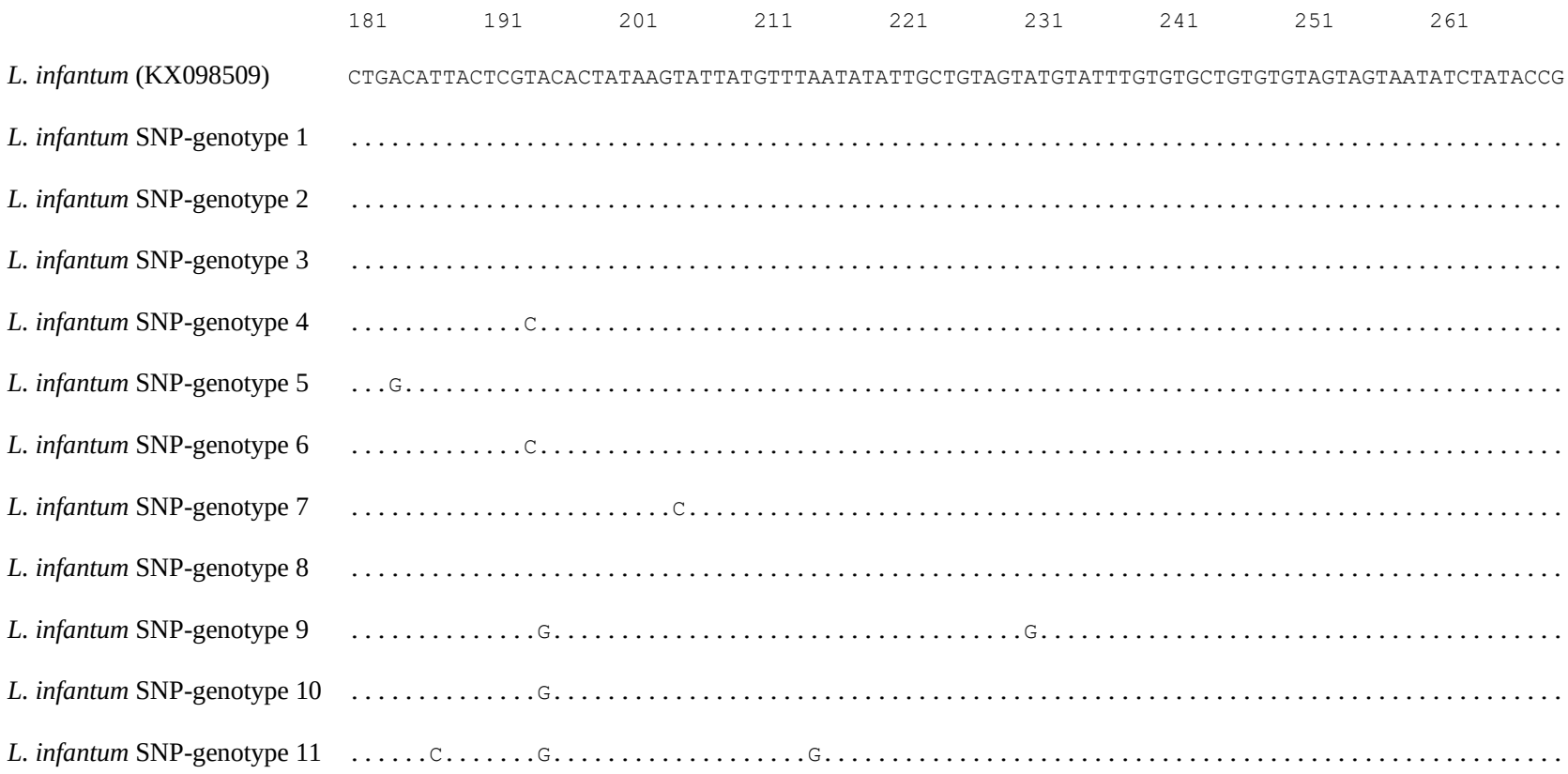
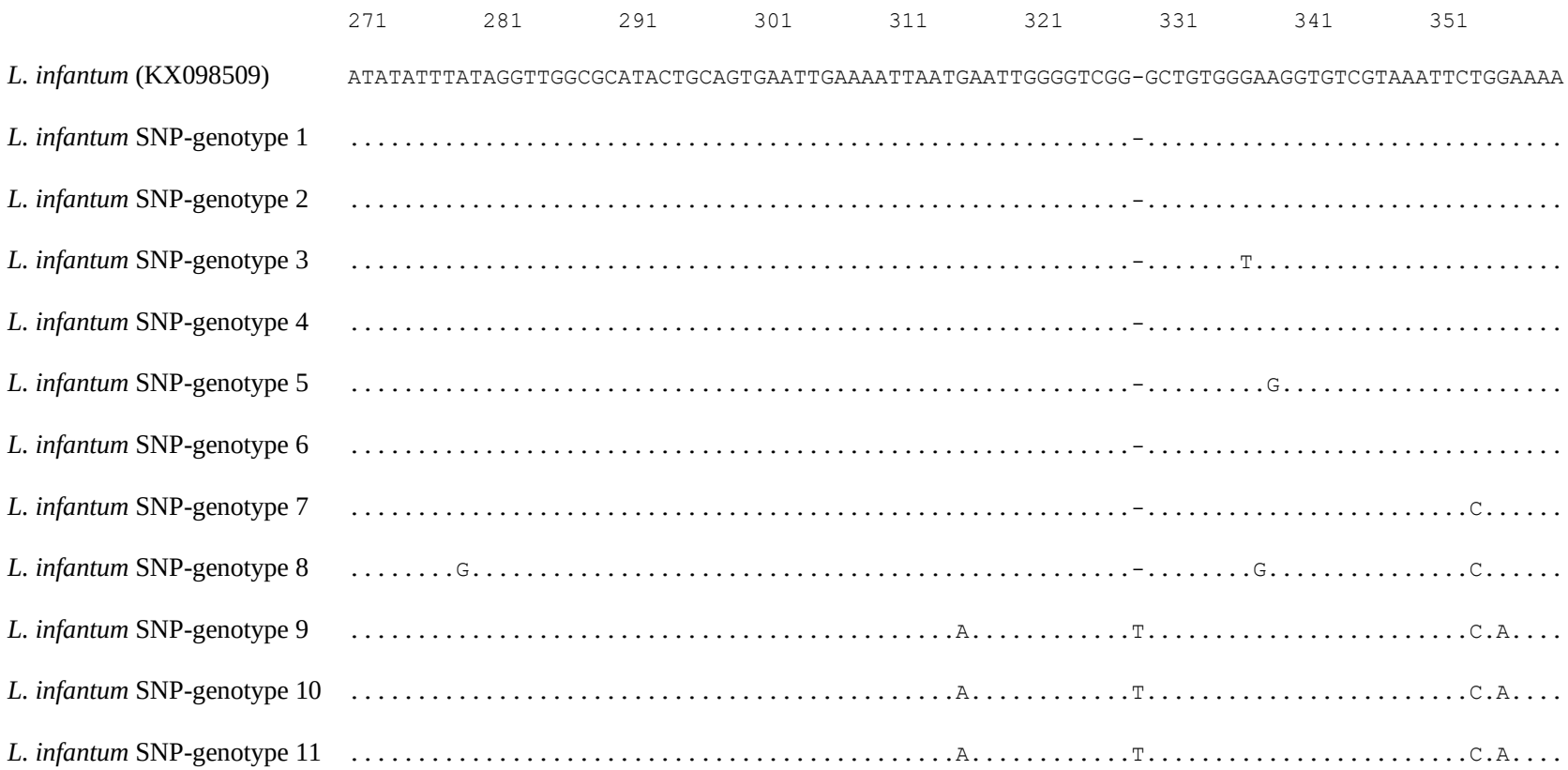
Supporting information

Table S1. Alignment of *Leishmania infantum* kDNA sequences including the reference sequence (GenBank access reference: KX098509). Nucleotide identity in each position in relation to the sequence of *L. infantum* is indicated with a dot (.), indels are indicated with a dash (-).

	1	11	21	31	41	51	61	71	81
L. infantum (KX098509)	GTTAGCCGATGGTGGTCTTGGGTTGACCTTAGGTGGTGATTCTCTTTAATATTGATATTAAAGGTAAGTGCTTGGACATATGATATAGCC								
L. infantum SNP-genotype 1	.								
L. infantum SNP-genotype 2	.							-	
L. infantum SNP-genotype 3	.								
L. infantum SNP-genotype 4	.								
L. infantum SNP-genotype 5	.								
L. infantum SNP-genotype 6	.								G
L. infantum SNP-genotype 7	.			G					
L. infantum SNP-genotype 8	.			G					
L. infantum SNP-genotype 9	.			G					C
L. infantum SNP-genotype 10	.			G				-	C
L. infantum SNP-genotype 11	.			G					C


91 101 111 121 131 141 151 161 171
L. infantum (KX098509) ATAGCGCTTTAGAATAGTTCGACTCCGAAGACCCAGTCTGAGGTAGTGTTAAGTATACATTAATCTAGTATATAATTTAGCATATAATAA
L. infantum SNP-genotype 1 .....
L. infantum SNP-genotype 2 .....
L. infantum SNP-genotype 3 .....
L. infantum SNP-genotype 4 .....
L. infantum SNP-genotype 5 .....
L. infantum SNP-genotype 6 .....
L. infantum SNP-genotype 7 .....A.....G.....
L. infantum SNP-genotype 8 .....A.....G.....
L. infantum SNP-genotype 9 .....A..G.....
L. infantum SNP-genotype 10 .....A..G.....
L. infantum SNP-genotype 11 .....A..G.....


181 191 201 211 221 231 241 251 261
L. infantum (KX098509) CTGACATTACTCGTACACTATAAGTATTATGTTTAATATATTGCTGTAGTATGTATTTGTGTGCTGTGTGTAGTAGTAATATCTATACCG
L. infantum SNP-genotype 1 .....
L. infantum SNP-genotype 2 .....
L. infantum SNP-genotype 3 .....
L. infantum SNP-genotype 4 .....C.....
L. infantum SNP-genotype 5 ...G.....
L. infantum SNP-genotype 6 .....C.....
L. infantum SNP-genotype 7 .....C.....
L. infantum SNP-genotype 8 .....
L. infantum SNP-genotype 9 .....G.....G.....
L. infantum SNP-genotype 10 .....G.....
L. infantum SNP-genotype 11 ......C.......G.....G.....


271 281 291 301 311 321 331 341 351
L. infantum (KX098509) ATATATTTATAGGTTGGCGCATACTGCAGTGAATTGAAAATTAATGAATTGGGGTCGG-GCTGTGGGAAGGTGTCGTAAATTCTGGAAAA
L. infantum SNP-genotype 1 .....-.....
L. infantum SNP-genotype 2 .....-.....
L. infantum SNP-genotype 3 .....-.....T.....
L. infantum SNP-genotype 4 .....-.....
L. infantum SNP-genotype 5 .....-.....G.....
L. infantum SNP-genotype 6 .....-.....
L. infantum SNP-genotype 7 .....-.....C.....
L. infantum SNP-genotype 8 .....G.....-.....G.....C.....
L. infantum SNP-genotype 9 .....A.....T.....C.A....
L. infantum SNP-genotype 10 .....A.....T.....C.A....
L. infantum SNP-genotype 11 .....A.....T.....C.A....

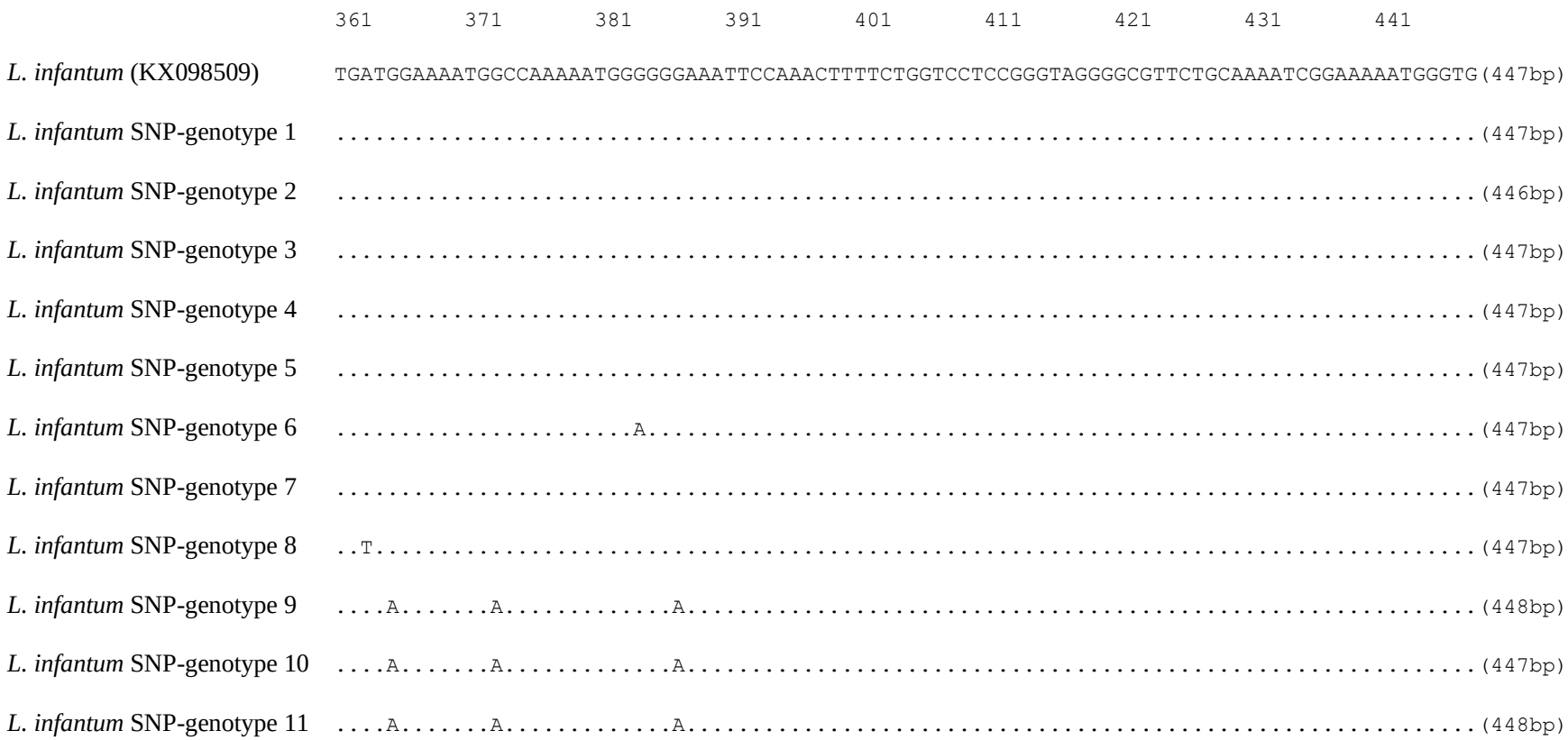

361 371 381 391 401 411 421 431 441
L. infantum (KX098509) TGATGGAAAATGGCCAAAAATGGGGGGAAATTCCAAACTTTTCTGGTCCTCCGGGTAGGGGCGTTCTGCAAAATCGGAAAAATGGGTG(447bp)
L. infantum SNP-genotype 1 .....(447bp)
L. infantum SNP-genotype 2 .....(446bp)
L. infantum SNP-genotype 3 .....(447bp)
L. infantum SNP-genotype 4 .....(447bp)
L. infantum SNP-genotype 5 .....(447bp)
L. infantum SNP-genotype 6 .....A.....(447bp)
L. infantum SNP-genotype 7 .....(447bp)
L. infantum SNP-genotype 8 ..T.....(447bp)
L. infantum SNP-genotype 9 ....A.....A.....A.....(448bp)
L. infantum SNP-genotype 10 ....A.....A.....A.....(447bp)
L. infantum SNP-genotype 11 ....A.....A.....A.....(448bp)

Table S2. A description of hosts and tissue samples on which the genetic characterisation of the kinetoplast minicircle of *Leishmania infantum* was performed, and the resulting RFLP (restriction fragment length polymorphism) and SNP (single nucleotide polymorphism) genotypes.

No. Sequence	Ind.	Host	Tissue	Sex	Age[1]	Season of collection	Geographical origin	HIV[2]	Clinical status	Clinical form	RFLP genotype	SNP genotype
1	1	Human	Bone marrow	M	33	Winter	Ghana	Neg.	Symptomatic	HVL[4]	B	G01
2	2	Human	Bone marrow	F	2	Spring	Spain	Neg.	Symptomatic	HVL	B	G01
3	3	Human	Bone marrow	M	48	Autumn	Spain	Neg.	Symptomatic	HVL	B	G01
4	4	Human	Bone marrow	M	29	Autumn	Spain	Neg.	Symptomatic	HVL	B	G01
5	5	Human	Bone marrow	M	46	Spring	Not- Spain	Neg.	Symptomatic	HVL	B	G01
6	6	Human	Bone marrow	M	51	Spring	Spain	Neg.	Symptomatic	HVL	S	G06
7	7	Human	Skin	F	47	Autumn	Spain	Neg.	Symptomatic	HCL[5]	F	G07
8	8	Human	Blood	M	41	Spring	Not Spain	NK	Symptomatic	HVL	F	G08
9	9	Human	Bone marrow	F	60	Autumn	United Kingdom	Neg.	Symptomatic	HVL	T	G09
10	10	Human	Skin	F	59	Winter	Spain	Neg.	Symptomatic	HCL	T	G10
11	11	Human	Bone marrow	F	5	Spring	Spain	Neg.	Symptomatic	HVL	T	G11
12	12	Dog	Spleen	F	9	Autumn	Spain	-	Symptomatic	CVL[6]	B	G01
13	12	Dog	Lymph node	F	9	Autumn	Spain	-	Symptomatic	CVL	B	G05
14	13	Dog	Spleen	F	4.5	Autumn	Spain	-	Asymptomatic	-	B	G01
15	14	Dog	Spleen	F	NK	Winter	Spain	-	Asymptomatic	-	B	G01
16	15	Dog	Spleen	F	3.5	Winter	Spain	-	Asymptomatic	-	B	G01
17	16	Dog	Spleen	M	5	Spring	Spain	-	Symptomatic	CVL	B	G01
18	16	Dog	Spleen	M	5	Spring	Spain	-	Symptomatic	CVL	B	G01
19	17	Dog	Spleen	NK[3]	6	Spring	Spain	-	NK	NK	B	G01
20	18	Dog	Spleen	M	6.5	Winter	Spain	-	Symptomatic	CVL	B	G01
21	18	Dog	Spleen	M	6.5	Winter	Spain	-	Symptomatic	CVL	B	G01
22	19	Dog	Spleen	M	4.5	Winter	Spain	-	Asymptomatic	-	B	G02
23	20	Dog	Spleen	F	NK	NK	Spain	-	Asymptomatic	-	B	G03
24	21	Dog	Spleen	M	NK	Winter	Spain	-	Asymptomatic	-	S	G04
25	22	Dog	Spleen	F	NK	Winter	Spain	-	Symptomatic	CVL	S	G06

Table S2 (continued). A description of hosts and tissue samples on which the genetic characterisation of the kinetoplast minicircle of *Leishmania infantum* was performed, and the resulting RFLP (restriction fragment length polymorphism) and SNP (single nucleotide polymorphism) genotypes.

No. Sequence	Ind.	Host	Tissue	Sex	Age[1]	Season of collection	Geographical origin	HIV[2]	Clinical status	Clinical form	RFLP genotype	SNP genotype
26	23	Rabbit	Liver	M	NK	NK	Spain	-	Asymptomatic	-	B	G01
27	24	Rabbit	Skin	F	NK	Winter	Spain	-	Asymptomatic	-	B	G02
28	25	Rabbit	Skin	F	NK	Winter	Spain	-	Asymptomatic	-	B	G02
29	26	Rabbit	Skin	F	NK	NK	Spain	-	Asymptomatic	-	B	G02
30	27	Rabbit	Skin	F	NK	NK	Spain	-	Asymptomatic	-	B	G02
31	28	Rabbit	Skin	F	NK	Winter	Spain	-	Asymptomatic	-	B	G02
32	29	Rabbit	Skin	F	NK	NK	Spain	-	Asymptomatic	-	B	G02
33	30	Rabbit	Spleen	F	NK	Winter	Spain	-	Asymptomatic	-	B	G02
34	31	Beech marten	Skin	F	Adult	NK	Spain	-	Asymptomatic	-	B	G02
35	32	Beech marten	Spleen	M	Adult	NK	Spain	-	Asymptomatic	-	B	G02
36	33	Beech marten	Spleen	M	Adult	Spring	Spain	-	Asymptomatic	-	B	G02
37	34	Genet	Skin	F	Adult	Summer	Spain	-	Asymptomatic	-	B	G02
38	35	Wolf	Skin	M	Adult	Spring	Spain	-	Asymptomatic	-	B	G02
39	36	Brown bear	Skin	NK	NK	NK	Spain	-	Asymptomatic	-	B	G02
40	37	Rat	Spleen	M	Young	NK	Spain	-	Asymptomatic	-	B	G02
41	38	Fox	Liver	M	Adult	Summer	Spain	-	Asymptomatic	-	B	G02
42	39	Fox	Spleen	M	Senior	Winter	Spain	-	Asymptomatic	-	B	G02
43	40	Fox	Spleen	F	Adult	Summer	Spain	-	Asymptomatic	-	B	G02
44	41	Fox	Spleen	M	Young	Autumn	Spain	-	Asymptomatic	-	B	G02

[1]Number refer to years.
[2] HIV: human immunodeficiency virus status
[3] NK: not known
[4] HVL: human visceral leishmaniosis
[5] HCL: human cutaneous leishmaniosis
[6] CVL: canine visceral leishmaniosis

GENERAL DISCUSSION

The epidemiological relevance of leishmaniosis caused by *Leishmania infantum* in human and animal hosts is increasing worldwide in the current context of globalisation, increased travelling, urbanisation and climate change. As a result, vectors populations are expanding towards more northern latitudes and higher altitudes, infected people and animals are bringing infection to areas that are not endemic for leishmaniosis but in some cases where vectors are present, and contact between the domestic and wildlife environments is increasing. Leishmaniosis deserves increased attention by the scientific community in order to find further ways of preventing and controlling infection.

This work was meant to close some important gaps in leishmaniosis epidemiology and to corroborate other scientific findings whilst procuring important information about this infection in southern Spain. We confirmed *Leishmania* infection in humans, domestic dogs and cats, and wildlife species including foxes, badgers, beech martens and Egyptian mongooses. Moreover, we provide some evidence of imported *Leishmania* species (*L. panamensis*) from a human patient from south America (Fernández-Arévalo et al., 2021). An important achievement of this thesis is the study of *L. infantum* infection prevalence and tissue distribution in cats (Chapter 1). Cats are increasingly being considered as a secondary reservoir of *L. infantum*, since a large proportion of cats may be infected in endemic countries (Pennisi et al., 2000; Chatzis et al., 2014; Maia et al., 2008; Millán et al., 2011). So far, no studies have demonstrated cat's ability to maintain *L. infantum* infection in the absence of dogs but xenodiagnostic experiments confirmed their ability to transmit the parasite to sandflies (Maroli et al., 2007). Also, they are present in very large populations in the domestic environment. The results of Chapter 1 show that *L. infantum* infection is common in stray cats, although prevalence is lower compared to dogs, which agrees with other studies from the same or similar areas (Tabar et al., 2008; Tabar et al., 2009; Otranto et al., 2017; Maia et al., 2010). We highlight the importance of the type of biological sample used for diagnosis to estimate the prevalence of infection. Parasitaemia is rare in healthy animals and PCR on blood is less sensitive than in tissue samples. Moreover, in contrast to dogs for which lymphoid tissue is the ideal sample for detecting infection, in cats, skin seems equally suitable as lymphoid tissue. The comparatively lower PCR prevalence in lymphoid tissue in cats compared to dogs may indicate a lower tendency for infection in cats to remain latent in reticuloendothelial tissue which is compatible with cats' lower risk of developing severe clinical disease and symptoms often being restricted to the skin. These findings are relevant from a diagnostic and clinical perspective and we

believe that it is important to contrast this hypothesis and generally to continue investigating the epidemiological contribution of cats to *L. infantum* transmission.

The finding that *L. infantum* is widespread in wildlife (Chapter 2) confirms previous studies. Moreover, the fact that strains maybe shared among wildlife, humans and dogs (Chapter 4) reinforces the idea that given the right epidemiological circumstances wildlife could represent a significant reservoir of *L. infantum* parasites and thus, an infection source for sandflies potentially biting humans and domestic animals, just like European rabbits and Iberian hares did in the community outbreak in Madrid (Arce et al., 2013; Molina et al., 2012; Jiménez et al., 2014). The list potential wildlife *L. infantum* reservoirs in Spain has increased after Egyptian mongoose were found to be infected for the first time (Chapter 2). Moreover, *L. infantum* infection in wildlife was significantly associated to *Hepatozoon* spp. and *Babesia* spp. infections although not in dogs and cats. This may be partly explained by differences in the risk of infection with these pathogens in domestic and sylvatic environments. In any case we consider important to further explore the immunopathological and clinical interactions between these pathogens for a better understanding of the relationship between *L. infantum* and *Hepatozoon* spp. and *Babesia* spp. As part of the *Hepatozoon* spp. research carried out, we discovered new host species for *Hepatozoon martis, Hepatozoon canis* and *Sarcocystis* spp., further contributing to the development of Veterinary Parasitology.

The study of *L. infantum* and *Phlebovirus* infections in blood donors (Chapter 3) provided important epidemiological information. On the one hand, *L. infantum* prevalence was lower in most places than in a similar study a decade earlier and also there were marked differences in the seroprevalence of *Phlebovirus* infections between samples taken two and half years apart at different times of the year. These results indicate important seasonal and interannual variation in infection intensity for both pathogens, that requires further investigation including entomological studies to examine vector infection rates. The study also reinforces the importance of considering blood samples from asymptomatic blood donors as a source of infection with *L. infantum*, and the need to maintain preventive treatments of donated blood to eliminate parasites prior to transfusion. Moreover, results also highlight the need for complementary studies on potential transplant-transmitted leishmaniosis, which is likely to represent a higher risk than blood transfusions since the load of *L. infantum* in organs such as liver is much higher than in blood, and no pathogen inactivation/reduction treatments are applied to organs.

Also, of epidemiological and clinical interest are the results on the spatial distribution of phleboviruses and *L. infantum* infections, which did not always coincide suggesting independent transmission cycles and different infection risk factors. Previous studies in Murcia Region have reported substantial differences between areas in the density of vectors. It would be useful to further characterise demographically and environmentally, areas with low and high prevalence of these sand fly-borne pathogens, and to estimate vector density and infection rates in these areas. Moreover, studies of the potential pathological mechanisms associated with coinfection with these pathogens merit further investigation. Although phleboviruses are not considered important pathogens of dogs and cats, studies in Portugal reported positive associations in these hosts species between Sicilian *Phlebovirus* (SFSV) seropositivity and clinical signs that were compatible with leishmaniosis (Pereira et al., 2019) Maia et al., 2017). Moreover Bichaud et al. (2011), reported a relationship between Toscana *Phlebovirus* (TOSV) and *L. infantum* in humans from southeastern France.

The risk of leishmaniosis and TOSV infections was found to be positively associated to the density of antibodies against sandfly saliva, which provide an indirect measure of exposure to sandfly bites. The role of salivary components in facilitating and impeding *Leishmania* spp. infection following parasite inoculation in the host by the vector has been studied in mice models. Saliva is considered to have both an "enhancing effect" whereby it exacerbates the infection by *Leishmania* spp. during primoinfections and a "protective effect" reducing infection following repeated exposure to sand fly bites (Lestinova et al., 2017; Rohoušová & Volf, 2006). These apparently contradicting effects are not considered mutually exclusive and it would be useful to investigate if similar effects occur in dogs and humans.

Results from Chapter 3 also provide useful diagnostic information. Human response to *Phlebotomus perniciosus* saliva components had not been investigated before, and we tested the *P. perniciosus* recombinant protein rSP03B as a practical alternative to salivary gland homogenate. The low to moderate correlation between them contrasts to similar studies in dogs where it was higher. The reasons for this are unknown and it was proposed that it could be related to differences in the immune response against salivary antigens between host species (Lestinova et al., 2017; Rohousova et al., 2005). Our results raise the question on whether there are other *P. perniciosus* salivary recombinant proteins that would be more suitable for diagnosing exposure to the vector. Investigating this issue should be

encouraged. Similar studies should also be carried out for *P. ariasi*, the other important vector species in Spain.

In summary, the work performed for this thesis highlights the complexity of sandfly and tick-borne infections in animals and humans and the clear need to approach research from a One Health perspective. Our findings provide a description of epidemiological and aetiological features of *L. infantum*, Phleboviruses, *Hepatozoon* spp. and *Babesia* spp. in some areas of southern Spain. Similar work needs to be carried out in other areas in order to generate risk maps for these important infections. They are the basis for developing sciences-based control strategies that reduce the impact of these pathogens.

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Conclusions

FIRST: *Leishmania infantum* infection is highly prevalent in stray cats and abandoned dogs from Murcia Region, and parasite body distribution differs between these host species. This further supports the potential implications of cats in the epidemiology of this parasite.

SECOND: *Hepatozoon canis* and *Hepatozoon felis* are widespread in dogs and cats, respectively, from Murcia Region. However, *Hepatozoon* spp. and *Leishmania infantum* infections are not associated in these host species.

THIRD: *Hepatozoon canis* and *Hepatozoon felis* are not specific to dogs and cats, respectively, and they also infect wildlife suggesting common transmission cycles.

FOURTH: The protozoans *Leishmania infantum, Hepatozoon* spp., and *Babesia* spp. are widespread among wildlife species, including foxes, badgers, beech martens and Egyptian mongooses, highlighting the potentially important role of wildlife in the epidemiology of infections by these pathogens.

FIFTH: There is a statistical positive association between *Leishmania infantum* and *Hepatozoon* spp. and *Babesia* spp. in wildlife hosts.

SIXTH: *Hepatozoon canis* is able to infect beech martens and *Hepatozoon martis* of infecting wild cats.

SEVENTH: Genets can be infected with *Sarcocystis* spp. and they could be the definitive host of *Sarcocystis cymruensis.*

EIGTH: Asymptomatic blood donors become infected with *Leishmania infantum* and Toscana phleboviruses and the risk of infection is directly related to exposure to the vector *Phlebotomus perniciosus*.

NINTH: Correlation between sandfly salivary gland homogenates and recombinant protein rSP03B antibody density is relatively low suggesting that the latter has limited value as a marker of exposure to sand fly bites.

TENTH: *Leishmania infantum* is genetically variable, some genotypes are specific for humans or animals whereas others are shared between them, supporting the existence of

anthroponotic and zoonotic transmission cycles, and the interaction between domestic and sylvatic contexts.

Conclusiones

PRIMERA: La infección por *Leishmania infantum* es altamente prevalente en gatos callejeros y perros abandonados de la Región de Murcia, y la distribución corporal del parásito varía entre estas dos especies de hospedadores. Esto apoya aún más las posibles implicaciones de los gatos en la epidemiología de este parásito.

SEGUNDA: *Hepatozoon canis* y *Hepatozoon felis* están muy extendidos en perros y en gatos, respectivamente, de la Región de Murcia. Sin embargo, las infecciones por *Hepatozoon* spp. y *Leishmania infantum* no están asociadas en estas especies de hospedadores.

TERCERA: *Hepatozoon canis* y *Hepatozoon felis* no son específicos de perros y gatos, respectivamente, sino que también infectan a la fauna silvestre, sugiriendo ciclos de transmisión comunes.

CUARTA: Los protozoos *Leishmania infantum, Hepatozoon* spp. y *Babesia* spp. se encuentran extendidos entre las especies de fauna silvestre, incluyendo zorros, tejones, garduñas y meloncillos, destacando el papel potencialmente importante de la fauna silvestre en la epidemiología de las infecciones por estos patógenos.

QUINTA: Hay una asociación positiva estadísticamente significativa entre *Leishmania infantum* y *Hepatozoon* spp. y *Babesia* spp. en hospedadores de la fauna silvestre.

SEXTA: *Hepatozoon canis* es capaz de infectar garduñas y *Hepatozoon martis* de infectar gatos monteses.

SÉPTIMA: Las ginetas pueden estar infectadas con *Sarcocystis* spp. y podrían ser el hospedador definitivo de *Sarcocystis cymruensis.*

OCTAVA: Los hemodonantes asintomáticos se infectan con *Leishmania infantum* y el *Phlebovirus* Toscana, y el riesgo de infección está directamente relacionado con la exposición al vector *Phlebotomus perniciosus*.

NOVENA: La correlación entre las densidades ópticas frente al homogenado de glándulas salivares y a la proteína recombinante rSP03B es relativamente baja, sugiriendo que ésta

última tiene un valor limitado como marcador de la exposición a la picadura de los flebotomos.

DÉCIMA: *Leishmania infantum* es genéticamente variable, algunos genotipos son específicos de humanos o animales mientras que otros son compartidos entre ambos, apoyando la existencia de ciclos de transmisión antroponóticos y zoonóticos, y la interacción entre los contextos domésticos y silvestres.

APPENDICES

Appendix 1. Scientific publications derived from this doctoral thesis

Articles

Ortuño, M., Latrofa, M. S., Iborra, M. A., Pérez-Cutillas, P., Bernal, L. J., Risueño, J., Muñoz, C., Bernal, A., Sánchez-Lopez, P. F., Segovia, M., Annoscia, G., Maia, C., Cortes, S., Campino, L., Otranto, D., & Berriatua, E. (2019). Genetic diversity and phylogenetic relationships between *Leishmania infantum* from dogs, humans and wildlife in south-east Spain. *Zoonoses and Public Health*, *66*(8), 961–973. https://doi.org/10.1111/zph.12646

Ortuño, M., Nachum-Biala, Y., García-Bocanegra, I., Resa, M., Berriatua, E., & Baneth, G. (2021). An epidemiological study in wild carnivores from Spanish Mediterranean ecosystems reveals association between *Leishmania infantum*, *Babesia* spp. and *Hepatozoon* spp. infection and new hosts for *Hepatozoon martis*, *Hepatozoon canis* and *Sarcocystis* spp. *Transboundary and Emerging Diseases*. Advance online publication. https://doi.org/10.1111/tbed.14199

Communications at scientific meetings

Ortuño, M., Risueño, J., Muñoz, C., Bernal, A., Maia, C., Cristovão, JM., Pereira, A., Campino, L., & Berriatua, E. (2017). Genotipado de cepas de *Leishmania infantum* utilizando kDNA-PCR-RFLP. Oral communication. III Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain).

Ortuño M., Risueño J., Muñoz C., Bernal A., Ortiz J., Maia C., Cristovão J.M., Pereira A., Cortes S., Campino L., & Berriatua E. (2017). Intraspecific diversity of *Leishmania infantum* from human, domestic and wild animal hosts. Oral communication. EurNegVec (COST) Final Meeting, Crete (Greece).

Muñoz, C., Ayhan, N., Risueño, J., **Ortuño, M.,** Ortiz, J., Charrel, R.N., & Berriatua, E. (2017). Seroprevalence of Toscana and Sicilian virus in healthy blood donors from rural areas in southeast Spain. Poster. EurNegVec (COST) Final Meeting, Crete (Greece).

Ortuño, M., Risueño, J., Annoscia, G., Muñoz, C., Goyena, E., Latrofa, M.S., Otranto, D., & Berriatua, E (2018). Relaciones filogenéticas entre aislados de *Leishmania infantum* procedentes de humanos, perros y fauna salvaje de España. Oral communication. IV Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain).

Ortuño, M., Risueño, J., Annoscia, G., Muñoz, C., Goyena, E., Latrofa, M.E., Otranto, D., & Berriatua, E. (2018). Unravelling relationships between *Leishmania infantum* infecting humans, dogs and wildlife from South-eastern Spain. Oral communication. 1st International Caparica Congress on Leishmaniosis, Lisbon (Portugal).

Ortuño, M., Risueño, J., Annoscia, G., Muñoz, C., Goyena, E., Latrofa, M.E., Otranto, D., & Berriatua, E. (2018). Unravelling relationships between *Leishmania infantum* infecting humans,

dogs and wildlife from South-eastern Spain. Poster. 1st International Caparica Congress on Leishmaniosis, Lisbon (Portugal).

Ortuño, M., Risueño, J., Annoscia, G., Muñoz, C., Goyena, E., Maia, C., Cortes, S., Latrofa, M.S., Campino, L., Otranto, D., & Berriatua, E. (2018). Genetic diversity of *Leishmania infantum* suggest a common transmission cycle in humans, dogs and wildlife in Southeast Spain. Poster. The 15th International Symposium of Veterinary Epidemiology and Economics, Chiang Mai (Thailand).

Ortuño, M., Resa, M., García-Bocanegra, I., Camacho, L., Jiménez-Ruíz, S., Caballero-Gómez, J., Cano-Terriza, D., & Berriatua, E. (2019). Resultados preliminares sobre la prevalencia de *Leishmania infantum* en animales silvestres del sur de España. Oral communication. V Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain).

Ortuño, M., Resa, M., García-Bocanegra, I., Camacho, L., Jiménez-Ruíz, S., Caballero-Gómez, J., Cano-Terriza, D., & Berriatua, E. (2019). Prevalence of *Leishmania infantum* in wild animals from southern Spain. Oral communication. XXI Congreso de la Sociedad Española de Parasitología (SOCEPA) e Intereuropeo de Parasitología, Murcia (Spain).